CHLOROTHALONIL.
ITS ENVIRONMENTAL FATE,
TOXICOLOGY AND METABOLISM
IN FISH

by

Peter Eric Davies B.Sc. (Hons.)

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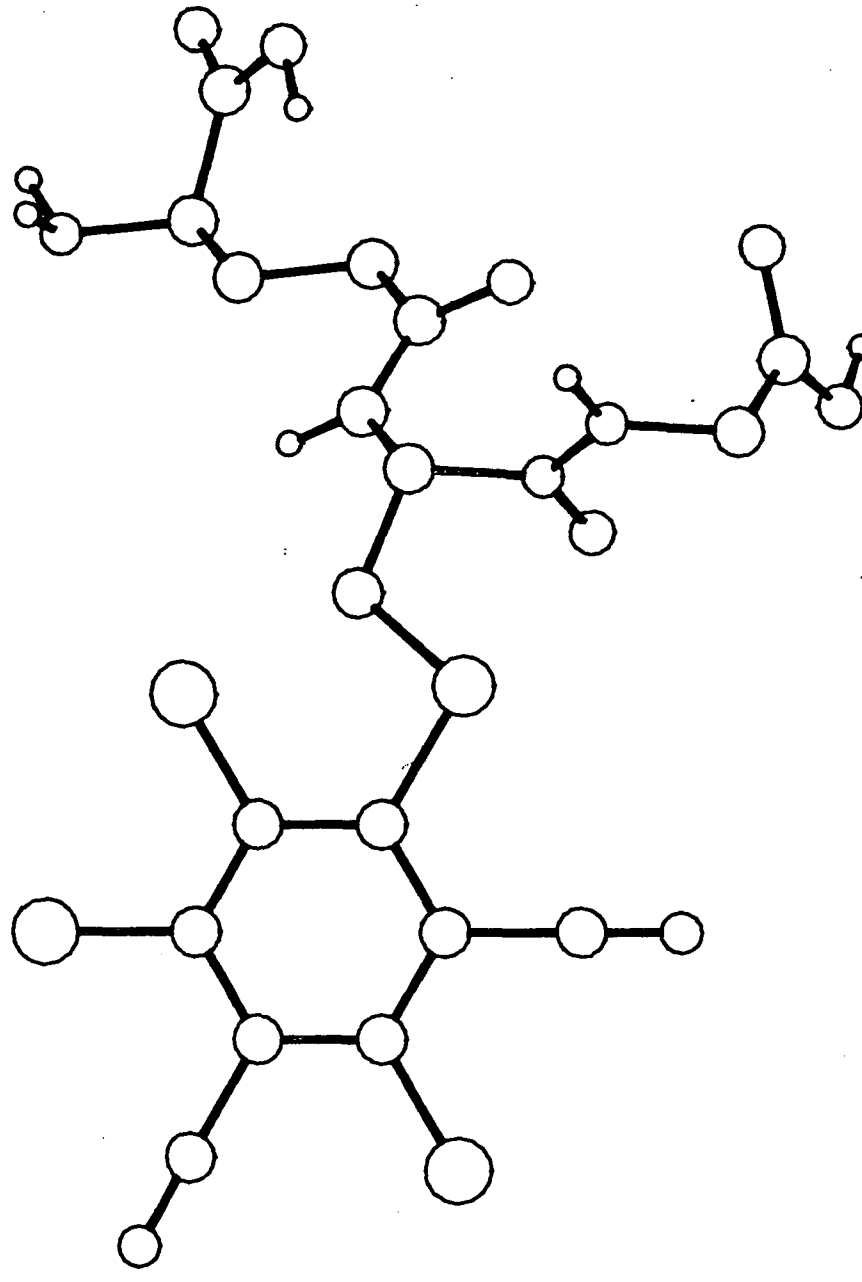

ORTEP plot of the structure of
4-(glutathione-S-yl)-2,5,6-trichloro-isophthalonitrile

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SUMMARY

The toxicology of a widely used fungicide, chlorothalonil (TCIN), was investigated in three species of native Australian galaxiid fish and rainbow trout, Salmo gairdneri Rich., after measuring environmental levels in two aerially sprayed north-eastern Tasmanian catchment streams. TCIN residues (0 - 5 ug/l) were detected intermittently in stream waters after spraying events. LC50 values determined in flow through tests were from 12 - 21 ug/l. Investigation of sublethal effects showed short term anaemia, haemolysis and long-term gill damage quantified by morphometric analysis. No anaerobic glycolysis was measurable on short-term lethal exposure, despite a synergistic effect of low oxygen levels measured in an LC50 test at 50% saturation. A device counting opercular rhythms by Fast Fourier Transform analysis was designed and tested in conjunction with experiments to determine respiratory rate and responses of S. gairdneri to low TCIN levels. A distinct respiratory response was measured, characterized by elevated ventilation rates, with a threshold of 20 ug/l during 2 h exposure. Environmental degradation of TCIN was studied in stream waters with a variety of substrates, temperatures and water types. TCIN was found to be a non-persistent pollutant whose disappearance rates were markedly enhanced by the presence of algal substrate and the presence of fish. Sediment partition coefficients were established. In a stream-dosing study, TCIN was found to be highly associated with suspended sediment.

In order to study metabolism of chlorothalonil (TCIN) in more detail, a synthetic route was designed and used successfully to produce (C^{14}-CN) TCIN. Tracing experiments were carried out in 10 ug/l exposed rainbow trout. Significant levels built up in all organs. Protein binding occurred in the liver. The primary concentration of label occurred in the gall bladder (up to 4000 ug/g in 96h). Sephadex filtration and TLC showed that glutathione conjugates were the primary metabolites. Structural studies were carried out using synthesized model compounds, and the structures of TCIN glutathione conjugates were fully elucidated by spectral methods.

Catalysis of the conjugation reaction by hepatic glutathione S-transferase (GST) enzymes was studied in detail in the three Galaxias

species, S. gairdneri and S. trutta. Molecular weights, comparative activities to another substrate (CDNB), pH optima, induction by TCIN exposure, binding and inhibition, reaction order, and organ specificity were all studied. All species were found to have two GST enzymes of different molecular weight with peak activity toward the two substrates, C^{14}-TCIN and CDNB. Galaxias GST's were consistently lower in molecular weight than those of the salmonids, indicating a major phylogenetic difference in this enzyme group. Induction and binding of metallothioneins by TCIN and co-induction with Zn exposure were also studied. Hepatic GST activity was promoted in S. gairdneri, G. maculatus and G. truttaceus exposed to low levels of TCIN over four days, and the response was dose dependent. Confirmation of detoxication by glutathione (GSH) depletion was obtained by measuring GSH levels in livers of exposed fish. Despite in vitro inhibition at low GAPDH levels, GAPDH inhibition was prevented by the presence of glutathione and was low in hepatic cytosol preparations of similar low GAPDH levels. Inhibition of GAPDH in livers of TCIN exposed fish was also studied, and found to be only transitorily affected by exposure to lethal levels.

No similar work on either toxicology, degradation or metabolism of pesticides in Australia has been reported in the literature. Apart from some heavy metal and hydrocarbon toxicology studies, almost no such work has been performed in Australia, most information being derivative from overseas. This study shows that the toxicological response of the galaxiids appears to be as sensitive as that of rainbow trout. A case is made for aquatic toxicological information applied within Australia to be directly related to information on native and not overseas species.

A proposal is made to screen native fish, both marine and freshwater, before time-consuming, expensive toxicity tests, possibly detrimental to native species populations, are carried out. The screening process involves selecting species "at risk" to classes of pollutants by studying levels and activities of detoxication enzyme systems. This proposed procedure is illustrated with the example of a correlation between GST and GSH levels in Salmo gairdneri, Galaxias auratus, and Galaxias maculatus, and the LC50 values for TCIN for these species, determined in this work.

A paper on the synthesis of C^{14}-TCIN has been accepted for publication. The manuscript is contained in Appendix 5. Six other papers have been submitted for publication on the toxicology and metabolism of chlorothalonil in fish.

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Many foreign, or xenobiotic, chemicals ultimately find their way into the aquatic environment and an understanding of their effects, fate and metabolism is essential with regard to the safety and stability of natural aquatic ecosystems and, less directly, of man (Khan et al., 1979). Studies of the fate and effects of foreign compounds in the aquatic environment developed in a number of areas, especially during the 1970s.

Early studies dealt primarily with investigations of lethal response levels of toxicants to aquatic organisms and some time elapsed before set procedures for aquatic bioassays were proposed and generally adopted (Sprague, 1969, 1970; Standard Methods, 1975; Matthews, 1980). It was soon realised that studies of the chronic effects of low level exposures were essential, especially as most xenobiotics occur in water systems at low concentrations and often over long time periods. More sensitive biochemical and behavioural indices of the effects of pollutants were developed, leading to revisions of "acceptable" levels. Studies with fish have generally taken one of two directions.

Firstly, there are those which involve investigations of the effect of xenobiotics on a fish's physiological and metabolic state with these changes being used as indicators of water quality (Price, 1979). These include studies of responses of respiratory functioning (Morgan, 1975; Spoor et al., 1971; Cairns and Garton, 1982), coughing response (Carlson and Drummond, 1977; Carlson, 1982), cardio-vascular parameters (Bass and Heath, 1977; Majewski et al., 1978), and general activity (Cairns and Waller, 1971; Slooff, 1979). Also included are studies of biochemical indicators of metabolic activity such as brain Ache activity (Rabeni and Stanley, 1979), GPT and GOT activity (Gingerich, 1982), plasma ion levels (Matthiessen, 1981), and blood hemoglobin levels (Travis and Heath, 1980). Behavioural studies have investigated the avoidance response (Sprague, 1968; Schumacher and

Ney, 1980), changes in rheotaxis (Dodson and Mayfield, 1979), and selection of salinity (Hansen, 1972). Growth changes have also been used to indicate pollutant stress (Webb and Brett, 1972; Ryan and Harvey, 1977).

Many of these studies relate response and exposure levels to standard toxicological parameters such as the 24, 48 or 96 h LC50 values for the toxicant. Few have directly related laboratory studies to environmental levels of xenobiotics, or have even measured them. Since many researchers work with concentrations much higher than actually occur in the environment, such work could be criticised as being of purely academic interest. Experimenters now also realise that, since many environmental parameters interact with the toxicosis state, studies of the effects of temperature, oxygen levels, pH, and salinity on the chronic and lethal toxicity of a xenobiotic are required to fully relate laboratory studies to reality. Some attempts at linking aquatic toxicological studies with particular cases or aspects of environmental contamination have been made by Sprague et al. (1971), Lockhart et al.(1977), Rabeni and Stanley (1979), Payne and May (1979), Schumacher and Ney (1980), Matthiessen (1981) and Hildebrand et al. (1982), involving studies of chronic toxicological and ecological effects, residue levels and behavioural changes.

Secondarily, there are studies, parallel to those in mammalian pharmacology and toxicology, which investigate the transformation of xenobiotics in aquatic organisms. These have shown that fish possess the ability to metabolise a wide variety of foreign compounds by means of detoxication enzymes of broad specificity (Chambers and Yarborough, 1976). The types of compounds studied in aquatic toxicology are numerous, and reflect the great diversity of pollutants that occur in the aquatic environment. They include heavy metals, pesticides, industrial pollutants and effluents as well as acid conditions and suspended solids (Alabaster and Lloyd, 1982).

A review of the literature has confirmed some 17 classes of metabolic processes involving xenobiotics in fish, based on functional group reactions. They generally fall into the recognised divisions of phase I metabolic processes, centering around the microsomal cytochrome P450 monooxygenase system, and phase II metabolism involving processes of detoxication by conjugation. They are presented here in tabular form, categorised as metabolic reactions involving O, N, S and

halogen substituents or reactive groups. Functional group reactions are shown in schematized form. Where some information on the properties of the related enzyme systems exists, including rates, distributions and substrate preferences, references are supplied. Several illustrated examples are given for each, along with the species studied, noting that the review cannot be deemed exhaustive (Table 1.1).

A principal rationale behind the investigation of the toxicology and metabolism of xenobiotic compounds is that, by understanding these specific effects and processes, a greater degree of understanding about the general effects on the biota exposed to these chemicals will result. This, it is hoped, will lead to a more knowledgeable approach to management and control of release of xenobiotic compounds into the environment (Matthews, 1980). It is, therefore, surprising that few such studies are related to direct environmental measurements of exposure levels.

Pesticide release into the aquatic environment is frequently low-level, diffuse and sporadic, depending on a large number of variables which include rainfall, spraying methods and soil-water interactions. These interrelationships have been the subject of a considerable research effort aimed at modelling pesticide transport into ground and surface waters (Bailey et al., 1974; Wauchope and Leonard, 1978; Haith, 1980). Few studies have investigated the entry, levels, dispersal behaviour and fate of pollutants after release events such as agricultural spraying (Trichell et al., 1968; Eronen et al., 1979; Leonard et al., 1979; Pearce et al., 1979; Stanley and Trial, 1980). McKimm and Hopmans (1978) provide one of the few local australian examples.

It is often the case that incidents of aquatic environmental exposure to chemicals, as a result of agricultural spraying or industrial release, involve a number of toxins which may, or may not, act synergistically on organisms (Matthews, 1980). Lloyd and Jordan (1963) and Brown (1968) were among the first to recognise the possible interdependence of the effects of toxins when occurring together in the aquatic environment, and attempted to quantify the toxic effects of mixtures to the rainbow trout, Salmo gairdneri (Richardson). Since then, a number of studies have been made of the effects of exposure to more than one chemical on the ability of fish to metabolise and bioconcentrate them and the effects on survival, respiratory response,

enzyme induction and other parameters (Alabaster and Lloyd, 1982).

The environmental effects of xenobiotics are also strongly dependent on the persistence of these compounds and the toxicity of their breakdown products (Steward et al., 1967). Many organophosphate and carbamate pesticides, for example, show rapid breakdown in the field, in the contrast to highly persistent compounds like DDT, and the PCB's. The persistence of such materials is related to the ease of chemical breakdown, especially by hydrolysis, and of biochemical metabolism, especially by soil and sediment bacteria (Chakrabarty, 1982).

A second rationale for the study of toxicology and metabolism of xenobiotics in aquatic species is that by studying the mechanisms and enzyme systems of pharmacology in lower forms, such as fish, suitable model systems may be developed to enable the further understanding of higher animal systems. An excellent example is the comparative study of renal transport mechanisms (Forster, 1967; Pritchard and Miller, 1980). Similar to this in potential are the studies of fish immune defence systems (Smith, 1980) and blood-brain exchange pharmacokinetics (Fenstermacher, 1980). The use of S. gairdneri embryos as whole animal model systems for experimental carcinogenesis - a process involving detailed study of enzyme system activity and metabolic activation/deactivation of xenobiotics, particularly aflatoxin B_1 (see section III(a) in the following review), primarily via the cytochrome P450 MFO system - is described and recommended by Hendricks et al. (1980). However, a more detailed study of such toxicological and metabolic processes in fish is required to fully develop such model systems.

Research into the effects of xenobiotic materials on fish has generally fallen in three areas:

Toxicological - lethal and chronic condition responses.

Behavioural - behavioural and sensory responses.

Metabolic - detoxication process functioning and determination of metabolites.

These three areas are actually different parts of a spectrum of

response levels shown by an organism to a xenobiotic chemical. Toxicological research is primarily involved with overt effects of a pollutant which are detrimental to the fish. It ranges from the extreme response of individual and population death, to more subtle changes in the physiological activities of the organism such as alterations in blood constituents and decreases in normal cellular metabolic processes, and biochemical changes such as enzyme inhibition. The study of behavioural processes has centred around overt individual responses of fish to pollutant exposure which can act as "indicators" of a toxic state.

Metabolic studies generally involve responses at the purely secondary biochemical level. It is a matter of debate whether the production of metabolites of xenobiotic compounds is describable as detoxication and hence, in essence, "deliberate" (Remmer, 1977), or whether such processes are products of an accidental identity of reactive group functionality with that of already existing functionalities. Over a long period xenobiotic metabolism may be evolutionarily enhanced in efficiency by the natural selective pressure of toxic exposure. However, the great diversity of environmental and cellular contaminants, which may be inhibitory to normal primary metabolic enzymes, necessitates that any such enzyme-mediated xenobiotic metabolism show broad specificity toward general functional group or reactive classes of pollutants (Jakoby, 1982). Man now appears to be the primary producer of inorganic and organic pollutants, often with a high degree of biotic selectivity (Burnside et al., 1971). Present attempts in pesticide chemistry to improve target specificity are laudable (Hedin, 1982), although it is doubtful if effects on off-target organisms will ever be eliminated. This is particularly true in cases where highly potent compounds are already widely in use and are of great efficacy toward the target such as the pyrethrins and their analogues, which, unfortunately, are particularly toxic to fish (Malhotra et al., 1981).

Man is certainly bringing greater toxic selective pressures to bear on the aquatic biota. Whether the effects will lead to permanent evolutionary changes is unlikely, due to the transient nature of pollution by any one chemical, especially as the variety of pesticides and industrial byproducts increases. This may, therefore, lead to nothing more than a general degradation of the aquatic environment.

However, at least in one case, it has been shown that selective changes may result. Gambusia affinis populations in heavily contaminated areas were found to have a much higher resistance to organochlorine insecticides than populations in uncontaminated areas. McCorkle et al. (1979) showed that this hundred-fold increase in resistance was concomittant with a higher oxygen stress limit, thereby indicating at least one possible detrimental secondary effect.

A number of fish populations, such as Fundulus heteroclitus have induced levels of microsomal MFO activity in contaminated waters (Burns, 1976). Such an effect may well be a precursor to selective differentiation of populations by environmental exposure to xenobiotics.

Australian aquatic toxcicology is in its infancy. Little or no research into the effects of environmentally dispersed chemicals, other than heavy metals, on aquatic fauna has been published. Such work as is currently taking place consists of either simple residue analysis or basic lethal toxicology studies. As a consequence, Australia is in a unique position, since researchers can use many techniques which have only been developed overseas during the past decade.

The working group on Water Research Policy (AWRC, 1982) recently submitted a report on proposed directions for water research. On the subject of water quality criteria it states that:

> "the many beneficial uses of water are formally protected by water quality criteria and standards. Criteria and standards in Australia are largely derivative and are becoming increasingly out-dated. Much more effort is required to correct this situation than is presently being exerted;"

On the subject of environmental degradation it states that:

> "pollution has previously been identified as a prominent issue with regard to current and emerging water problems in the general area of catchment and land use. It, and other environmental impacts, now greatly affect many Australian inland waters in a deleterious fashion. Research on the causes and precise nature of, and possible remedial actions for, the continuing degradation of the aquatic environment constitute yet another research gap identified in several submissions."

The lack of research in Australia into the fate and effects of xenobiotic materials, especially organic compounds, possibly results from the use of "largely derivative" standards for water quality by

the Australian authorities. The role of the Commonwealth Department of Health in the assessment and control of pesticides, does not appear to cover the aquatic environment (Commonwealth Department of Health, 1978). LD50 data are required for "at least two unrelated species", and all data required are related to mammalian toxicology. No requests for toxicity testing in off-target organisms are made by the Poisons Scheduling Committee, which deals with registration of pesticides for marketing in Australia. The Pesticides and Agricultural Chemicals Sub-Committee of the National Health and Medical Research Council is required only to make recommendations on problems associated with maximum residue limits of pesticides and agricultural chemicals in food.

There are no legal requirements for compounds that can enter the aquatic environment to be tested for toxicity to australian freshwater or marine organisms. Derivative information regarding american and european fish species is often available although, in the light of present doubts about U.S. Environmental Protection Agency practices, it may be of dubious value. Such information may be relevant to introduced salmonid and carp stocks. However, all other aquatic species are effectively ignored and, in consequence, unprotected. Australia has about 180 known native fresh water fish species. Approximately half of these occur in eastern, southern and Tasmanian seaboard streams which are probably exposed to agricultural and industrial chemicals to varying degrees. There is no information available on the toxicology of these compounds to any of these species. The marine biota are equally neglected, although here the degree of exposure and, consequently, the relevance of particular studies is less easily defined.

1.2 PROJECT BACKGROUND

In 1980 the owner of a northern Tasmanian trout-farming company producing meat fish and eggs for australian and overseas markets, contacted the University of Tasmania voicing concerns about pollution in the Brid River and Muddy Creek, streams which supplied water to two separate trout-farms. He suggested that spraying of pesticides within the catchments may be causing inhibition of growth,

stress and death in farm trout during the summer months.

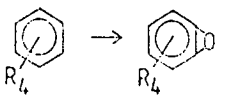
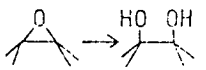
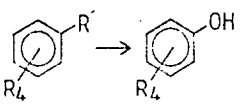
The local Department of Agriculture supplied a comprehensive list of pesticides known to be used in the area (Appendix 1). No information could be supplied as to their relative importance in relation to quantities used. The list comprised 23 chemicals and a means was sought to select primary suspects. Aerial spraying was regarded as the most likely cause of stream contamination. On inspection of material at the Bridport airstrip, a short list of aerial sprays was made (Appendix 1). Communication with a spraying company and local distributors revealed that regular intensive aerial spraying was carried out each year during the period mid-December to mid-March on a fortnightly basis.

On the basis of published data on the toxicity of the sprayed chemicals and other considerations detailed in the following chapter, it was decided to study the fate and effects of Chlorothalonil, or tetrachloroisophthalonitrile (TCIN), in the aquatic environment with particular emphasis on its toxicological effects on metabolism in several australian freshwater fish species. The study was used to examine if toxicological and metabolic data derived for Salmo gairdneri Richardson, a species of global distribution and intensively used as a research animal overseas, was comparable with data derived for species of native australian fish in the family Galaxiidae. This research was performed in the context of measured environmental levels of TCIN and likely residence times within the stream environment.

Table 1.1 Review of organic xenobiotic metabolic transformations reported in fish, grouped according to functionality.

A. General classes of metabolic processes.

B. Reported examples.

A	Group Reaction	Phase	Enzyme Systems	References to enzyme system properties
I. OXYGEN				
a. Aryl oxidation		I	Multifunction oxygenase Aldrin epoxidase Monooxygenase	Ahokas(1979); Bend et al.(1979);Melancon et al.(1981); Schoenhard(1974); Stott and Sinnhuber(1979); Schnell et al.(1980); Koivusaari et al.(1981).
b. Hydration		II	Epoxide hydrase	Bend and James(1978); Gregus et al.(1983).
c. Hydrolysis	RCONHR → RCOOH+NH_2 ArO(P) → ArOH RNRR → HNRR+ROH RCOOR → RCOOH+ROH	II	--	--
d. Hydroxylation		II	Aryl hydrocarbon hydroxylase	Ahokas(1979); Bend et al.(1979); Payne and May(1979).
	X = H, NO_2			
	$R_3CHal.$ → R_3COH		--	--
e. O-dealkylation	(P)OAlk. → (P)OH	II	O-demethylase	Miyamoto et al.(1979).
f. Glucuronide conjugation	ArOH → ArOgluc. $ArNH_2$ → ArNHgluc.	II	Glucuronyl transferase	Lech and Statham(1975); Elcombe et al.(1979); Gregus et al.(1983).
g. Sulphate conjugation	ArOH → $ArOSO_3H$	II	Sulphotransferase	Kobayashi(1979); Gregus et al.(1983).
h. Taurine conjugation	$ArCH_2COOH$ → $ArCH_2COta.$	II	--	--

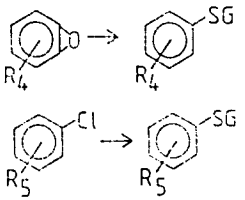
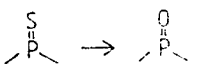
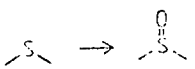
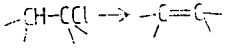
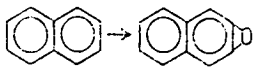
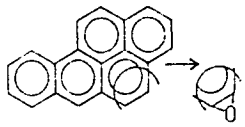
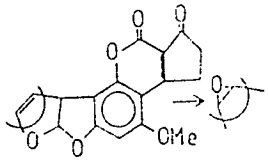
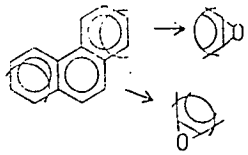
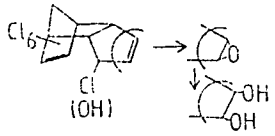
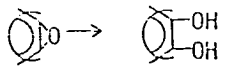
II. NITROGEN				
a. N-dealkylation	$RNHAlk \rightarrow RNH_2$	II	--	Olson et al.(1977).
b. N-acetylation	$RNHR \longrightarrow RNRCOCH_3$	II	Acetyl transferase	Allen et al.(1979); Gregus et al.(1983).
c. Reduction	$ArNO_2 \longrightarrow ArNH_2$	--	--	--
III. SULPHUR				
a. Glutathione conjugation		II	Glutathione epoxide transferase G S-transferase	Stott and Sinnhuber(1979). Nimmo et al.(1979,1981); Sugiyama et al.(1981); Bauermeister et al.(1983); Gregus et al.(1983).
b. Mercapturate generation	$RSG \rightarrow RSCH_2CHNHCOCH_3$ (COOH)	II	γ-glutamyl transpeptidase (see also N-acetylation)	Bauermeister et al.(1983).
c. S-methylation	$ArX \rightarrow ArSH \rightarrow ArSMe$	II	--	--
d. Oxidative desulfuration		--	--	--
e. Sulfoxidation		I	Multifunction oxygenase	Lay et al.(1979).
IV. HALOGEN				
a. Dehydrochlorination		--	--	Zinck and Addison(1975); Addison and Zinck (1977).

Table 1.1 B

1 Compound 2 Reaction	Fish species	References
Ia. Aryl oxidation		
Naphthalene 	1 Gillichthyes mirabilis 2 Oligocuttus maculosus 3 Citharichthys stigmaeus 4 Oncorhynchus kisutch 5 Clupea harengus pallasi	Lee et al.(1972) Lee et al.(1972) Lee et al.(1972) Roubal et al.(1977) Malins et al.(1979)
Benzopyrene 	1, 2, 3	Lee et al.(1972)
Aflatoxin B1 	6 Salmo gairdneri	Stott and Sinnhuber(1979); Hendricks et al.(1980).
Phenanthrene 	6 7 Platichthyes flesus 8 Squalus acanthias	Solbakken and Palmork(1981) Solbakken and Palmork(1981) Solbakken and Palmork(1981)
Ib. Hydration		
Heptachlor (Hydroxychlordane) 	9 Carassius auratus	Khan et al.(1979)

PCB,PBB,PAH's

1 - 8 — As above



Ic. Hydrolysis

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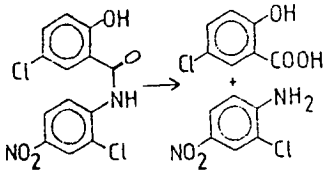
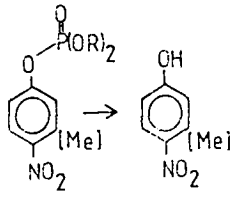


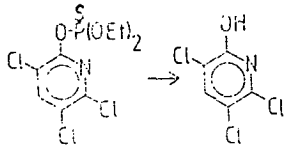
10 Cyprinus carpio — Allen et al.(1979)

Parathion(Et);Methylparathion(Me)
[Fenitrooxon]



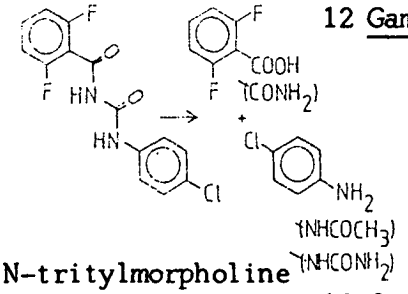
11 Lepomis gibbosus — Benke et al.(1974)
6, 10 — Miyamoto et al.(1979)

Dursban



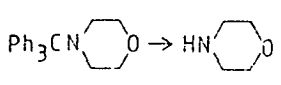
10 — Smith et al.(1966)

Diflubenzuron



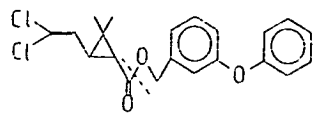
12 Gambusia affinis — Metcalf et al.(1975)

N-tritylmorpholine



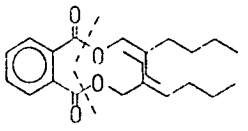
13 Sarotherodon mossambicus — Matthiessen et al.(1977)

Permethrin(cis,trans)



6, 10 — Glickman et al.(1979)

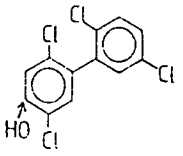
Phthalates(DEHP)



14 Fathead minnow — Stalling et al.(1973)
15 Ictalurus punctatus — Stalling et al.(1973)
6 — Melancon et al.(1976a,1977); Melancon and Lech(1979).
16 Cyprinodon variegatus — Wofford et al. (1981)

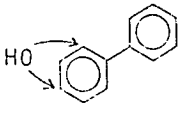
Id. Hydroxylation

PCB



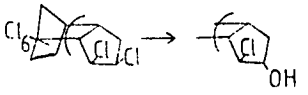
6 — Melancon and Lech(1976)

Biphenyl

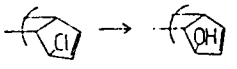


17 Skate — Creaven et al.(1965)
6 — Willis and Addison(1974)

cis-chlordane (heptachlor)

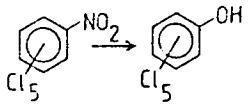


9 — Khan et al.(1979)
18 Cichlasoma sp. — Khan et al.(1979)
19 Lepomis macrochirus — Khan et al.(1979); Sudershan and Khan(1980).



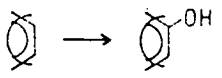
9 — Khan et al.(1979)

Quintozene



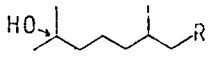
Bahig et al.(1981)

PCB,PBB,PAH's,TCDD



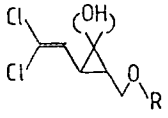
20 Raja erinacea — Bend et al.(1979)

Methoprene



19 — Quistad et al.(1976)

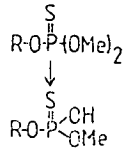
Permethrin



6, 10 — Glickman et al.(1979)

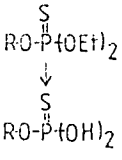
Ie. O-dealkylation

Fenitrothion



6, 10 — Miyamoto et al.(1979)

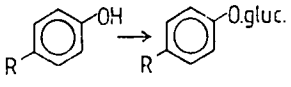
Dursban



9 — Smith et al.(1966)

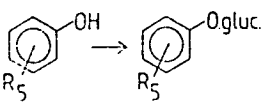
If. Glucuronide conjugation

Phenol red



21 Squalus acanthias — Bungay et al.(1976)

Phenol(H),PCP(Cl)



6 — Kobayashi(1979)

9 — Kobayashi(1979); Glickman et al.(1977); Layiwolla and Linnecar(1981).

22 Abramis brama — Layiwolla and Linnecar(1981)

23 Poecilia reticulata — Layiwolla and Linnecar(1981)

24 Phoxinus phoxinus — Layiwolla and Linnecar(1981)

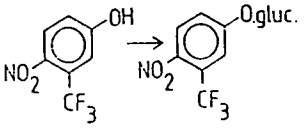
25 Perca fluviatilis — Layiwolla and Linnecar(1981)

26 Rutilus rutilus — Layiwolla and Linnecar(1981)

27 Scardinus erythrophthalmus — Layiwolla and Linnecar(1981)

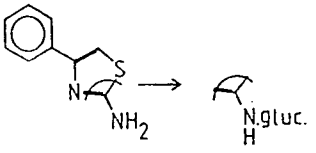
28 Tinca tinca — Layiwolla and Linnecar(1981)

TFM



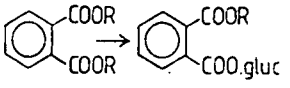
6 — Lech and Costrini(1972); Lech(1973); Hunn and Allen(1975a,b).

Piscaine



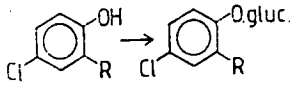
6, 10 — Suzuki et al.(1977)
29 Oryzias latipes — Suzuki et al.(1977)

DEHP



14, 15 — Stalling et al.(1973)
6 — Melancon and Lech(1976a); Melancon et al.(1977).

Bayer 73

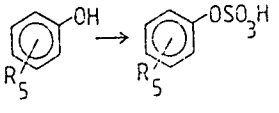


6 — Statham and Lech(1975); Allen et al.(1979).
30 Micropterus salmoides — Schultz and Harman(1980)

Fenitrothion,PCB — As above

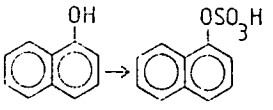
Ig. Sulphate conjugation

Phenol(H),PCP(Cl)



6, 9 — Kobayashi(1979)
9, 22 - 28 — Layiwolla and Linnecar(1981)

Naphthalene

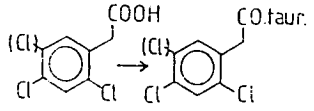


4 — Malins et al.(1978)
5 — Malins et al.(1979)
31 Parophrys vetulus — Varanasi et al.(1978)
32 Platichthys stellatus — Varanasi et al.(1978)
33 Lepidosetta bilineata — Varanasi et al.(1978)

Assay substrates	6	Gregus et al.(1983)

Ih. Taurine conjugation

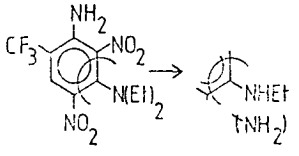
2,4-D (2,4,5-T)



	21	James and Bend(1976); Guarino et al.(1977).
	34 Pseudopleuronectes americanus	James and Bend(1976)

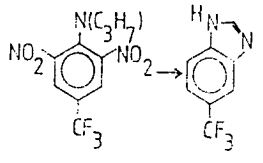
IIa. N-dealkylation

Dinitramine



	10	Olson et al.(1977)

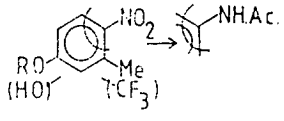
Trifluralin



	19	Isensee et al.(1979)

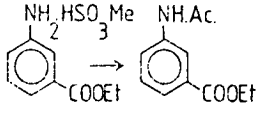
IIb. N-acetylation

Fenitrothion (TFM)



	10	Miyamoto et al.(1979)
	6	Lech and Costrini(1972): in vitro

MS222



	6	Hunn et al.(1968)
	21	Maren et al.(1968)

Piscaine

$RNH_2 \longrightarrow RNH.Ac.$

	6, 10, not in 29	Suzuki et al.(1977)

IIc. Reduction

Fenitrothion, TFM, Quintozene

$ArNO_2 \longrightarrow ArNH_2$

10 — As above.
6 — As above.
35 Golden Orf — Bahig et al.(1981)

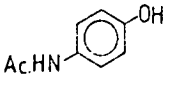
IIIa. Glutathione conjugation

CDNB

$ArCl \longrightarrow ArSG$

6 — Nimmo et al.(1979,1981); Bauermeister et al.(1983): in vitro.

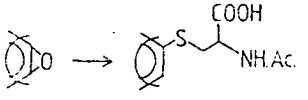
Acetaminophen



6 — Parker et al.(1981): in vitro. By inference from mercapturates.

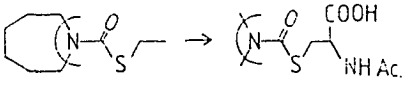
IIIb. Mercapturate generation

PAH epoxides



1 - 8 — As above

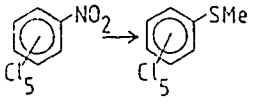
Molinate



10 — Lay et al.(1979)

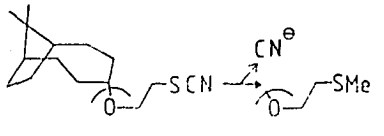
IIIc. S-methylation

Quintozene



35 — Bahig et al.(1981)

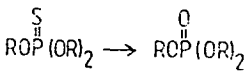
Thanite



10 — Allen et al.(1980)

IIId. Oxidative desulfuration

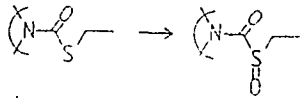
Parathion(A),Methylparathion(B),
Fenitrothion(C),Dursban(D).



11	(A) Benke et al.(1974)
6, 10	(B) Miyamoto et al.(1979)
6, 10	(C) Miyamoto et al.(1979)
10	(D) Smith et al.(1966)

IIIe. Sulfoxidation

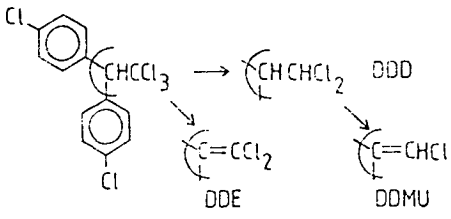
Molinate



10 Lay et al.(1979)

IVa. Dehydrochlorination

DDT



6		Addison and Willis(1978)
10		Yahalomi and Perry(1981)
36	Tilapia aurea	Yahalomi and Perry(1981)
37	Ctenopharyngodon idella	Yahalomi and Perry(1981)
34		Pritchard et al.(1973)
38	Salmo salar	Addison et al.(1976)
39	Solea solea	Ernste and Goerke(1974)

CHAPTER 2

ENVIRONMENTAL BEHAVIOUR OF CHLOROTHALONIL

2.1 ENVIRONMENTAL ANALYSIS AND LEVELS

2.1.1 INTRODUCTION

In order to fully assess the parameters leading to the observed stress behaviour of farm Salmo gairdneri at Bridport and Muddy Creek, it was decided to investigate a range of water quality parameters including environmental pesticide levels within the two catchments. As stated in Chapter 1, aerial spraying of pesticides was suspected as a possible cause of fish stress. It was found that intensive fortnightly spraying operations within the catchment areas of both trout-farm feeder streams was carried out during summer.

Two pesticides were sprayed in a mixed formulation: Bravo$^{(R)}$, a fungicide produced by The Diamond Shamrock Corporation, U.S.A., and Orthene$^{(R)}$, an insecticide produced by ICI, Australia. They were sprayed on potato crops throughout north-eastern Tasmania, only being applied in fine weather. All other pesticides observed at the Bridport airstrip, used to service the whole N-E region, were sprayed on an occasional and irregular basis.

Spraying sites within the adjacent Brid River and Muddy Creek catchments were numerous and concentrated, occurring mainly in the rich basalt soil country at the upper end of the catchments (Fig. 2.1). The topographical vegetative and geographical details of the area derived from Pinkard (1980) are shown in Fig. 2.2 and listed in Table 2.1. Stream flows were not found to be high, typically 0.15 - 0.2 m^3/s, 0.05 m^3/s and 0.1m^3/s in summer for the Brid River, Cox's Rivulet and Muddy Creek respectively. The upper tributaries (Fig. 2.1, marked - -) were found to be dry from December to March and little or no surface drainage occured during this summer period. Irrigation and watering of potato crops was common, leading to some drainage into the larger tributaries.

Both main streams flow through poor, sandy soil with heath-forest

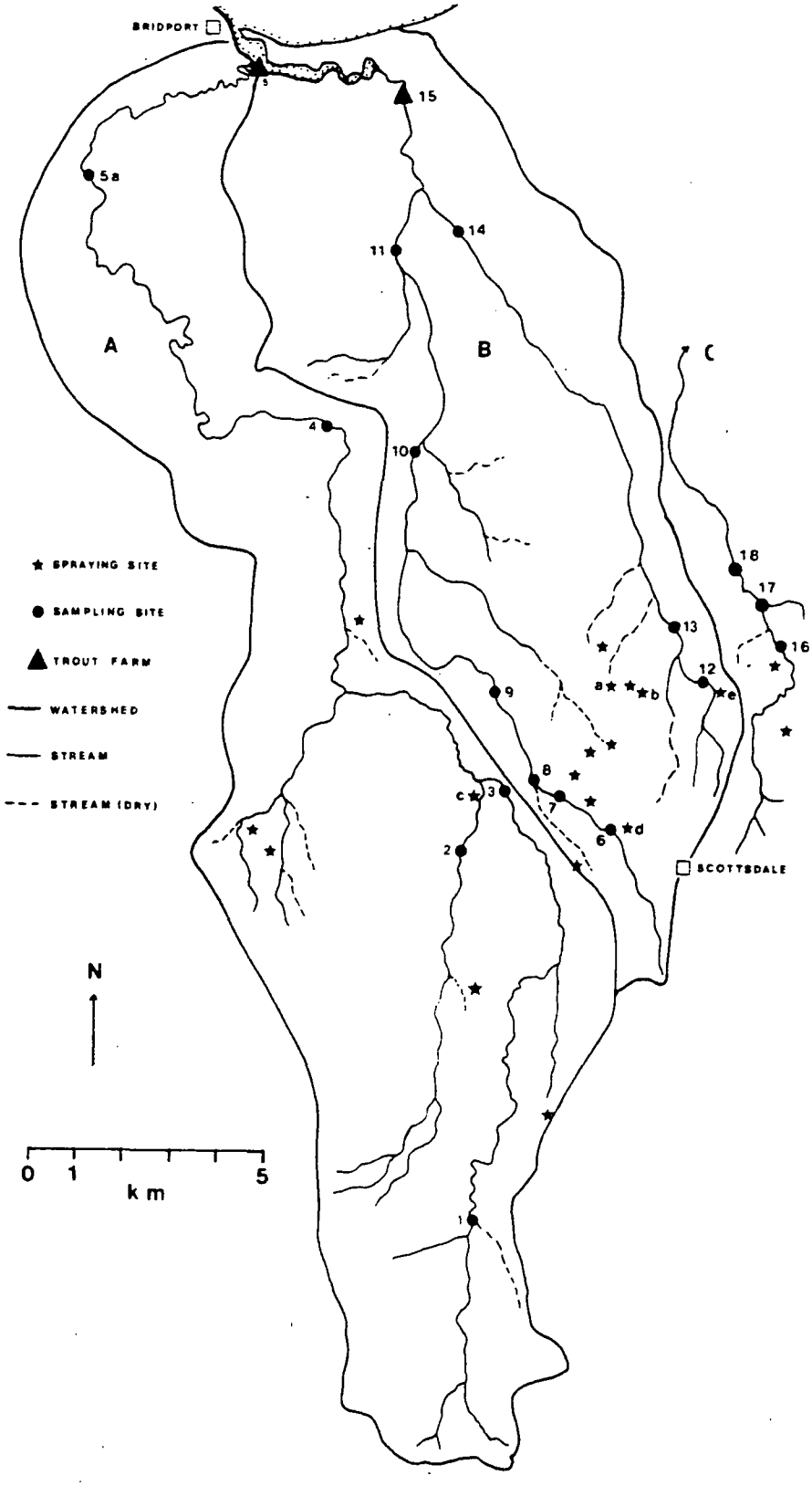


Fig. 2.1 Map of Bridport Study Area.

A = Brid River catchment serving Bridport farm.
B = Cox's Rivulet catchment, serving Muddy Creek farm.
C = Tucker's Rivulet catchment.
Aerial spraying sites are marked. Stream condition indicated as in summer of 80-81 and 81-82.
1 - 5 = water quality sampling sites.

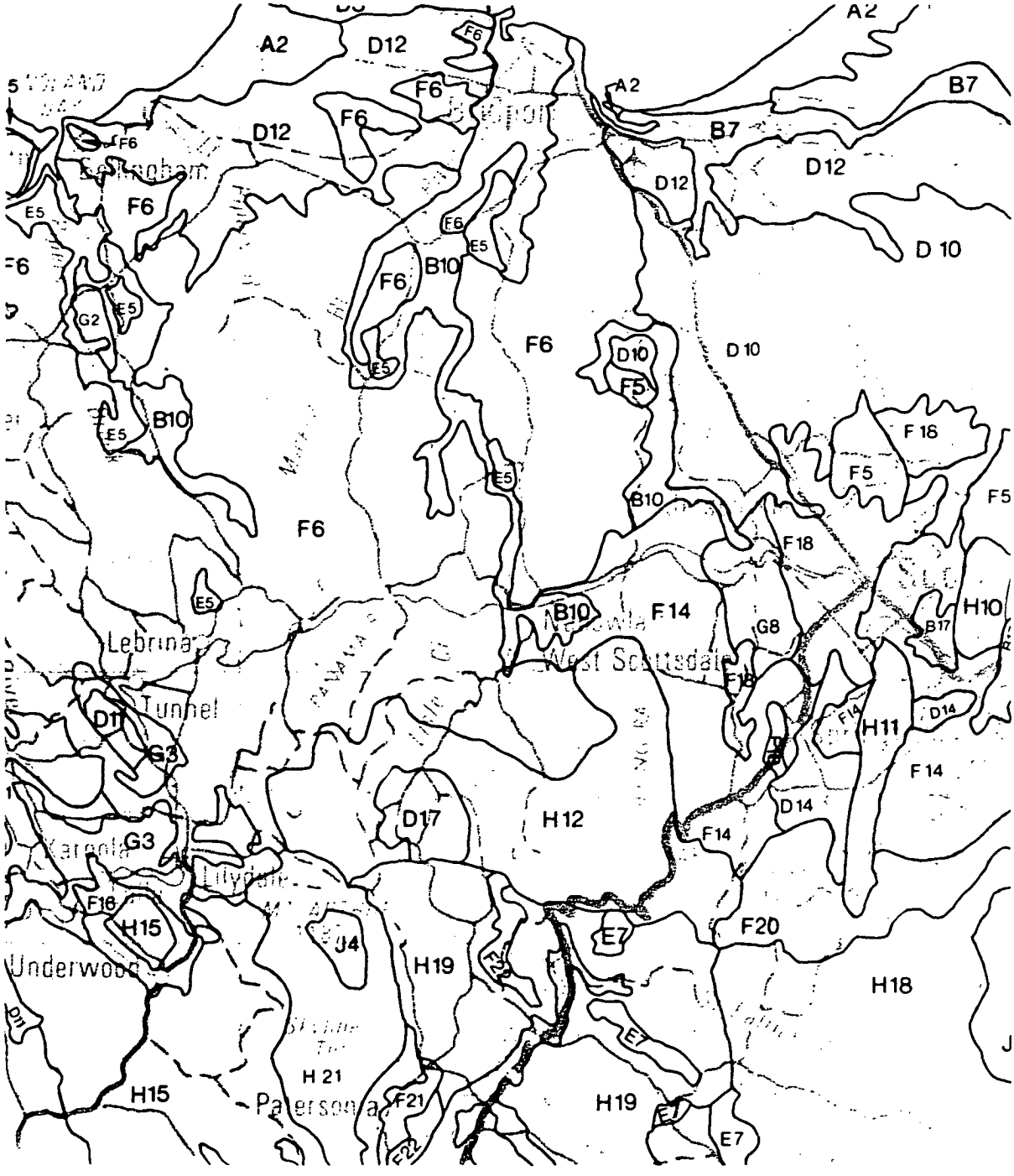


Fig. 2.2 Landforms within the study area. Reproduced from Pinkard (1980). See Table 2.1 for details.

Table 2.1 Land - form types within the Brid River and Muddy (Hurst) Creek - Coxs Rivulet catchments. Codes refer to topographical map in Fig. 2.2. Data from Pinkard, 1980.

Code	Landform type	Area code	Vegetation	Annual rainfall (mm)	Land use
B	Sand, clay and gravel. Woodland and scrub.	B7	None	625–750	None
		B10	Open shrubland	750–1000	Grazing
D	Eucalypt forested plains. Diverse sandy clay loams.	D10	Open-forest (Black peppermint)	750–1000	Grazing
		D12	Open-forest (Black peppermint)	750–1000	Grazing, forestry
E	Low hills. Stony or gravelly soil. Woodland	E5	Open woodland (White gum)	750–1000	Grazing, cropping
F	Eucalypt forested low hills. Yellowish red soils.	F5	Open-forest (Black peppermint)	750–1000	Grazing, forestry
		F6	Open-forest (Stringy bark)	750–1000	Softwood forestry, grazing
		F14	Open-forest (Stringy bark)	1000–1250	Grazing, forestry
		F18	Closed forest (White gum)	1000–1250	Grazing, cropping
G	Eucalypt forested low hills. Brown-grey gradational soils.	G8	Low open-forest (White gum)	1000–1250	Grazing

communities in their mid and lower reaches. This area is relatively undeveloped agriculturally due to its low fertility (Pinkard, 1980), and such development is pasture land. No other intensive pesticide spraying occurs within the catchments.

The literature contains few reports of the toxicity of chlorothalonil, or TCIN (tetrachloroisophthalonitrile), the active ingredient of Bravo (R), and of acephate (0,S-dimethyl acetyl phosphoroimidothioate), the active ingredient of Orthene (R), to fish. The toxicity of TCIN is, however, much higher than that of acephate (BCPC, 1979; Nishiuchi, 1977, 1979; Perevoznikov, 1977; Klaverkamp, 1982; U.S. Dept., pers. comm.). Typical reported values of LC50 for TCIN fall between 67 and 250 ug/l whereas for acephate values are all $\geqslant$ 1000 mg/l. Thus TCIN appears at least 4 - 15 x 10^3 times more toxic than acephate. It was considered unlikely that acephate would have any noteable effects on the farm trout, especially as the distance of the farms from the spraying sites was 15 - 20 km. Rabeni and Stanley (1979) reported only minor effects on fish in streams of spraying acephate in surrounding spruce forest at much higher rates than in the Brid and Muddy catchments. They found that slight transient depression of brain AchE activity occurred, but no significant effects on growth, condition or food consumption were observed. Consequently, it was decided to carry out a sampling and analysis program for TCIN in the catchment streams, and acephate was not investigated.

A sampling program was carried out in both catchments and trout-farms in order to detect possible factors that could have caused the symptoms observed by Purves in farm trout. Water quality parameters were measured in the farms to assess the water quality status of the ponds, and water samples were taken before and after an aerial spraying event in the summer of 1980-1981. Direct visual observation of spraying operations was also carried out, along with observations of trout behaviour in farm ponds.

2.1.2 MATERIALS AND METHODS

Sampling sites

Sampling sites in the catchment are shown in Fig. 2.1, samples being taken daily over several weeks, mid-stream in 250 ml acetone-rinsed glass and plastic bottles. Samples in the trout farms

were taken at the main inlet, pond inlets and outlets and mid-pond for both 0-1 and 1-2 year class ponds. Samples were collected at a number of sites in the catchment over the fortnight after a spraying event in early January 1981.

Water quality of catchment and pond waters

Temperature, oxygen and ammonia levels were assayed at 15 min to 1 h intervals through diurnal cycles and day-time feeding periods in the ponds of the Bridport farm. Oxygen was analysed by the Winkler method, azide modification (Standard Methods, 1975), or using a Yellow Springs International oxygen probe-meter. Ammonia was analysed immediately after collection by the phenate method (Standard Methods, 1975).

Other parameters measured at various sites in the catchment included nitrate, by the spectrophotometric method (Standard Methods, 1975), pH using a Radiometer pH probe and conductivity using a Radiometer CDM3 conductivity meter. Suspended solids were measured by filtering 1 l of water through a predried, preweighed glass fibre (Sartorius) filter. The filter was then dried to constant weight (60° C) and re-weighed.

Collection and analysis of samples for pesticide monitoring

Water samples, 250 ml, were collected in acetone-washed plastic bottles and frozen for storage. 240 ml of each sample was extracted with 10 ml redistilled hexane by magnetic stirring for 20 min. This process was 96% efficient by analysis of internal standards.

Dried (Na_2SO_4 anhyd.) hexane samples were analysed by gas chromatography on a Perkin Elmer 881 gas chromotagraph, 10% methane in argon gas as carrier, with 2% OV101/3% QF1 (1:1) on a Gaschrom Q 100-120 mesh column at 200° C. Typical retention times of around 4 min were obtained for chlorothalonil (TCIN), with a gas flow rate of 40 ml/min. Detection limit for TCIN = 0.001 ug/l, on a Pye electron capture detector used at 270° C.

Fish samples

Samples of fish muscle were taken from fish collected by Purves at times of fish kills during the summers of 1980-81 and 1981-82. They were homogenised with Na_2SO_4 (anhyd.) and extracted with hexane:acetonitrile (1:1, 5 vol., 3x). Samples were cleaned by passing through a

florisil column and eluted with hexane:ether (1:1). Analysis for TCIN was as above.

2.1.3 RESULTS

Typical results for conductivity, nitrate concentration, pH and suspended solids are presented in Fig. 2.3 for sites 1 - 5. A typical diurnal cycle of oxygen and ammonia levels is shown in Fig. 2.4 for 0-1 and 1-2 year ponds. Pond parameters and typical stocking rates are given in Table 2.2, along with typical oxygen levels and water quality parameters. Oxygen levels in the 1-2 year ponds were low from January to March, with mean mid-pond levels ranging from 3 to 6 mg/l. High stocking rates, low flows and high temperatures were also recorded for these ponds.

Signs of stress, usually manifested as crowding and 'finning' behaviour with surface gasping, were recorded only for fish in the 1-2 year ponds, and only during the period December to March. Not all stress periods were directly correlated, however, with high temperatures or particularly low oxygen concentrations. On three occasions they occurred within several days of aerial spraying operations in the upper catchment.

Analysis of water for chlorothalonil showed low levels occurring at sites indicated in Table 2.3. They all fell in the range 0-5 ug/l, and were chiefly less than 1.0 ug/l. All samples were taken during periods without rain, as rain did not fall until several weeks after the studied spray event in early January. The majority of samples, however, showed no detectable chlorothalonil levels.

Analysis of both water and live and dead fish samples taken during stress periods in the 1-2 year ponds showed no detectable chlorothalonil levels.

Chlorothalonil levels

Visual observation of spraying operations in 1981 and 1982 showed that all spraying was performed at very low altitudes and that drift was minimised. Orthene has a distinctive odour which served as a rough indicator of drift. On standing downwind from sites **a** and **b** during a light breeze, Orthene was detected up to 100 m downwind but

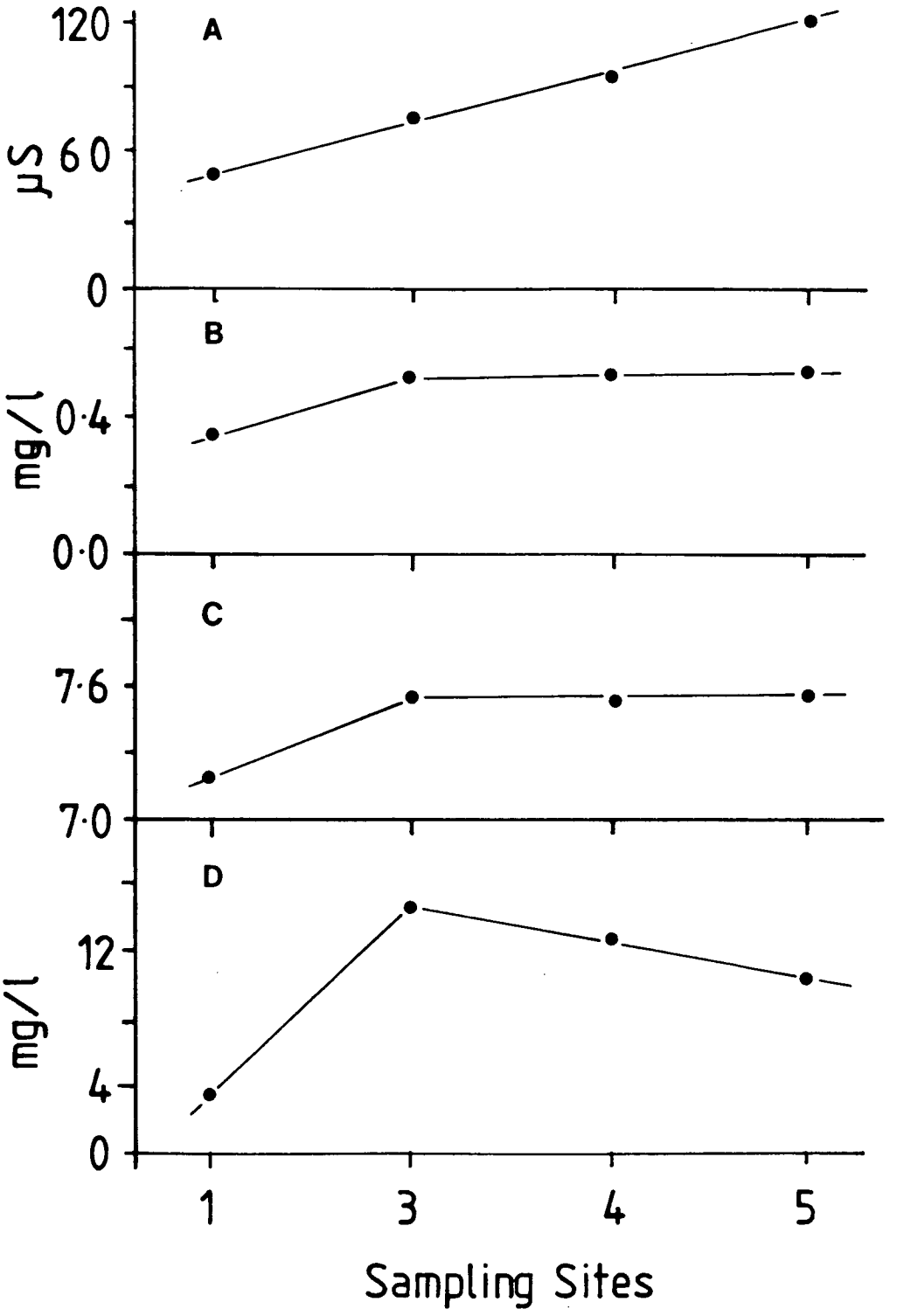


Fig. 2.3 Water quality of the Brid River (Summer 80–81).
A = Conductivity B = nitrate nitrogen,
C = pH D = suspended solids.
Sites are as shown in Fig. 2.1

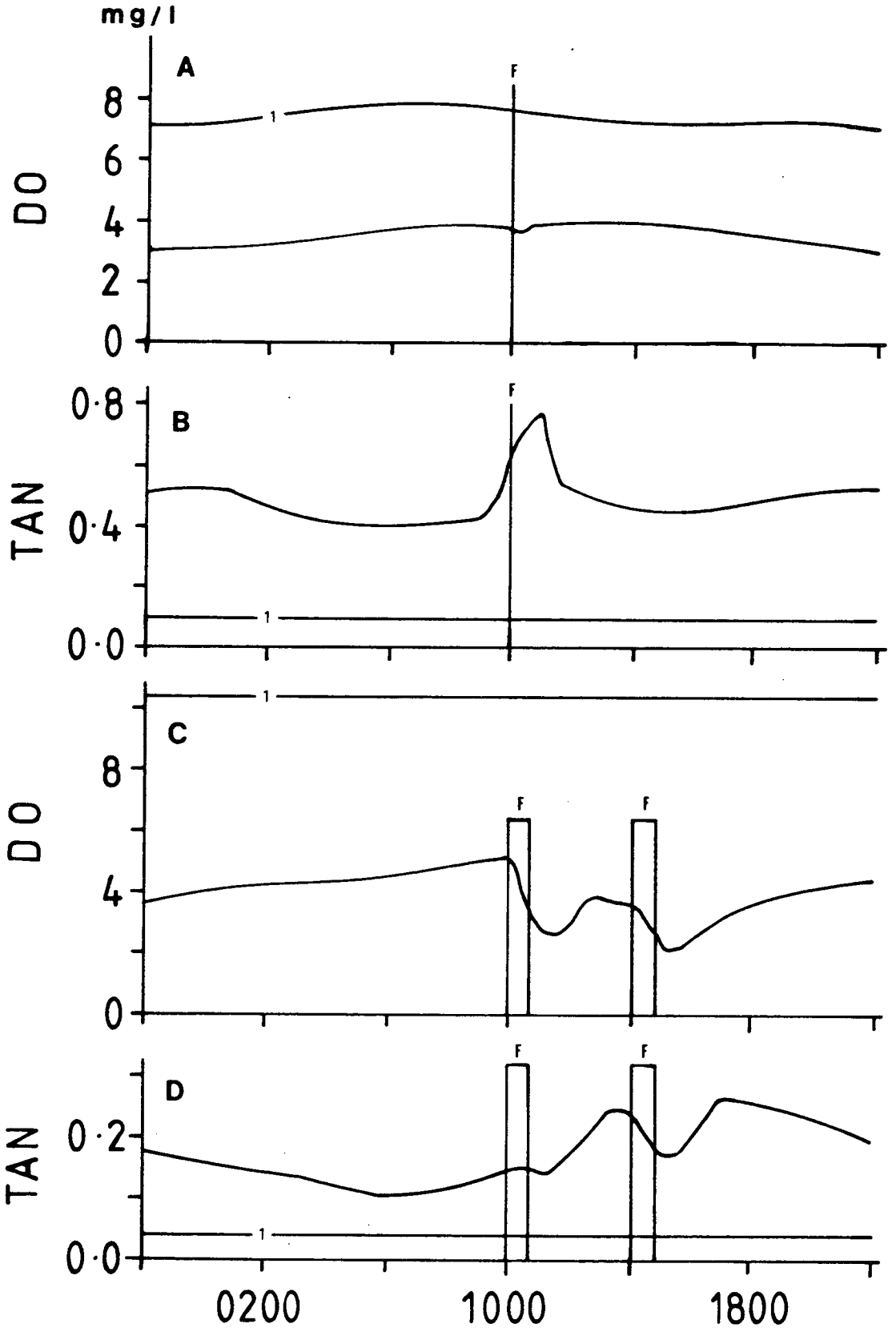


Fig. 2.4 Typical diurnal fluctuations in dissolved oxygen (DO) and total ammonia nitrogen (TAN) of inflows (1) and outflows of ponds at Bridport trout-farm, January to March (1980 - 1982).
A,B = 1 - 2 year ponds.
C,D = 0 - 1 year ponds.

Table 2.2 Pond parameters for the Bridport trout-farm

Parameters	Typical values
0 - 1 year ponds:	
Dimensions (m)	1.1x6.5x0.9; 2.3x14.5x0.9
Flow rates (m^3/s)	0.0098 - 0.020
Stocking rates (N, g)	7000 - 20000; 16 - 30
Summer DO, TAN (mg/l)	7 - 9; 0.1 IN
	5 - 8; 0.2 - 0.5 OUT
1 - 2 year ponds:	
Dimensions (m)	7x35x0.8
Flow rates (m^3/s)	0.045 - 0.090
Stocking rates (N, g)	4000 - 10000; 50 - 250
Summer DO, TAN (mg/l)	6 - 8; 0.2 - 0.3 IN
	3 - 5; 0.4 - 0.8 OUT

Water quality parameters:		IN	OUT
K25	(uS/cm)	159	155
Colour	(Pt u)	60	70
pH		5.24	6.77
Na	(uN)	870	896
K	(uN)	47.6	49.9
Ca	(uN)	56	12
Mg	(uN)	247	255
Cl	(uN)	890	898
HCO_3	(uN)	32	236
G_{440}	(m^{-1})	2.625	2.750

Summer temperatures	15 - 23° C

Table 2.3 Levels of chlorothalonil (TCIN) in stream waters of the catchments of the Brid River, Muddy Creek and Tuckers Rivulet after aerial spraying operations 1: at 1400 h, 25/2/1981, and 2: at 1600 h, 22/3/1981. See Fig. 2.1 for spraying and sampling site locations.

Spray event	Date	Sampling site	Time	Residue present?	[TCIN] (ug/l)
1	25/2	All sites	1400,1900	-	0.00
	26/2	7	1309	+	1.35
		12	1330	+	0.33
		10	1445	+	0.69
		All other sites	1200 - 1500	-	0.00
	27/2	All sites	0830 - 0930	-	0.00
	2/3,3/3,4/3	15	0900,1500	-	0.00
	5/3	15	0830	+	0.35
		15	1430	-	0.00
	6/3,7/3	15	0900,1500	-	0.00
2	22/3	12,13,14,16,17	0800,1600	-	0.00
	24/3	10,13,14,15,16,17	1700 - 1800	-	0.00
		12	1745	+	0.47
	25/3	16	1120	+	1.03
		17	1417	+	4.60
		18	1400 - 1600	-	0.00
		18	1457	+	0.78
	26/3 - 6/4	16,17,18	0800,1600	-	0.00

could not be detected elsewhere around the sites. All spray sites within the two catchments were at sufficient distances from active watercourses to eliminate direct drift as a cause of contamination, except for sites **c**, **d** and **e**.

2.1.4 DISCUSSION

No water quality parameter studied, except oxygen, was considered to occur at a level likely to cause stress in trout (Alabaster and Lloyd, 1982). Oxygen levels were low in the 1-2 year ponds. Alabaster and Lloyd (1982) recommended the 50 and 5 percentile lower limits of oxygen concentrations for salmonids of 9 and 5 mg/l respectively. They suggest minimum sustained dissolved oxygen levels of 3 and 4 mg/l for survival and juvenile growth, respectively. The minimum level of dissolved oxygen which can maintain maximum feeding, growth and efficiency of food conversion is 4-4.5 mg/l for rainbow trout at 10.5° C (Itazawa, 1971), and this is probably considerably higher at 15-20° C. Smith (1971) noted that the salmonid Oncorhynchus kisutch showed fatigue in active swimming behaviour after 2 h at 4-4.5 mg/l.

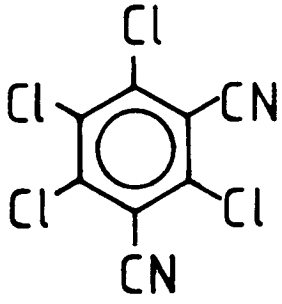
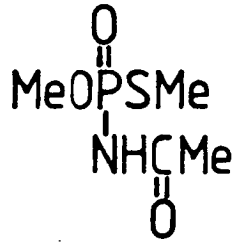
It therefore appeared that the mean summer oxygen levels experienced by Salmo gairdneri, at both the Muddy Creek and Bridport trout-farms, of 3-6 mg/l were suboptimal for growth, and at the lower end of the acceptable range, even for survival. It was considered that if TCIN contamination was causing a stress-related response in farm trout, that it would be as a synergistic reaction with the low oxygen levels. The fish, already under stress from low dissolved oxygen, may perhaps be very sensitive to the action of such pesticides as TCIN. TCIN levels in the order of 5 ug/l are around 0.02 - 0.1 of already published lethal levels for fish. Respiratory responses to pollutants often occur at fractions from 0.01 to 0.1 of the lethal level (Sloof, 1979). Sprague (1969) showed that S. gairdneri are sensitive to zinc at 0.01 of the lethal threshold. Such sensitivity could well be exacerbated under conditions of low oxygen, high temperatures and crowding. It is well established that the nature and magnitude of fish responses to pollutant organics are sensitive to other environmental parameters, especially temperature and low oxygen (Alabaster and Lloyd, 1982). In

subsequent chapters the existence of possible relationships between the toxic response to TCIN by Salmo gairdneri, low oxygen levels, and the respiratory system will be explored.

Aquatic environmental levels of pesticides after operational spraying have generally been found to be low. Despite agricultural runoff from herbicide sprayed crops containing levels ranging from 0 to 2000 ug/l, and being typically in the range 0 - 200 ug/l (Leonard et al., 1979), most studies have found that watershed streams are generally only contaminated in the low ug/l range, dependent on the extent of drift, time after spraying and the advent of rainfall (Rabeni and Stanley, 1979; Younos and Smolen, 1983). Background levels of organochlorines in non-polluted waters of Britain were in the order of 0 - 20 ug/l (Croll, 1969).

The occurence of low levels of pollutants in streams after spraying is a result of the complex set of organic matter - soil - water interactions which such organic materials experience during transport from the site of application to the watercourse. Considerable concentration losses occur due to microbial degradation and volatilization (Chakrabarty, 1982). The two pesticides, acephate and chlorothalonil, have quite different structures and physical properties (Table 2.4, BCPC, 1979). The high water solubility of the more polar acephate indicates that it is more likely to be carried at higher concentrations in first runoff than TCIN. TCIN is highly lipophilic and as such is more likely to be surface bound to organic material in soils (Chiou, 1981). Consequently, stream contamination of TCIN is more likely to be low-level and with longer entry and residence times. Acephate loss into ground and surface water is more likely to be rapid, and single-event. Acephate contamination is, therefore, expected to closely follow rainstorm events and have shorter entry and residence times than TCIN. The anticipated slow release behaviour of TCIN from sprayed watersheds may have profound effects on the environmental toxicology of the compound.

Table 2.4 Properties of chlorothalonil and acephate

	CHLOROTHALONIL	ACEPHATE
Structure		
Name	tetrachloroisophthalonitrile 2,4,5,6-tetrachloro-1,3-benzodinitrile TCIN, TPN	O,S-dimethyl acetyl-phosphoramidothioate ENT2822
Formulations	Bravo; Daconil 2787; Exotherm; Termil. (Diamond Shamrock Corp.)	Orthene (ICI)
M.P. (^{o}C)	250 - 251	82 - 89
V.P. (mm Hg)	0.01 (40^{o} C)	1.7×10^{-6} (24^{o} C)
Solubility (25^{o} C)		
- water	0.6 mg/l	650 g/l
- organic solvents	80 g/kg (xylene) 20 g/kg (DMSO) 20 g/kg (acetone)	<50 g/l >100 g/l
LC50 (*Salmo gairdneri*)	0.25 mg/l (estimate)	>1000 mg/l

Data from BCPC (1979).

2.2 BIODEGRADATION OF TCIN

2.2.1 INTRODUCTION

Organic compounds released into the environment are subjected to a variety of physical and biological conditions which cause a reduction in concentrations. They may undergo photolysis, volatilization and adsorption on surfaces or soil particulates. Once they have entered the soil column they may become absorbed to organic matter (Chiou, 1981) or bacterially degraded (Chakrabarty. 1982). On entering the water compartment, hydrolysis and volatilization may also occur, as well as diffusion and dispersion (Stanley and Trial, 1980; Roberts, 1983). Reaction with other chemical components of waters may also take place (Chapman et al., 1982).

The degradation of pesticides in the aquatic environment depends on the level of insolation, oxygen, bacterial flora and the pH of the water (Weinberger et al., 1982). Nutrients, such as glucose and yeast extract, as well as oxygen levels, were found to affect the degradation rate of PCP by influencing microfloral activity (Liu et al., 1981). The biodegradation rates of nitrilotriacetate in Rur River water were markedly affected by oxygen levels and temperature, indicating microbial action (Larson et al., 1981). The fate of fenitrothion in a small stream was studied by Moody et al. (1978). They found marked uptake by aquatic plants and water sediment, the latter 200 - 1000 times the water concentration. There was also evidence of microbial reductive metabolism. The increase in polarity of pesticides by microbial action and their subsequent increase in loss into the aqueous phase may be a significant factor in environmental detoxication (Weber and Rosenberg, 1980). In contrast, Nesbitt and Watson (1980 a,b) found no correlation between the degradation rate of 2,4-D and the microbial abundance in Western Australian River water.

In order to assess the environmental safety of a compound some measure of its biodegradability is required. There are standard methods for studying biodegradation, as outlined by Means and Anderson (1981), but these chiefly involve high activity, enriched media suitable only for comparative studies. In order to assess the behaviour of a compound in the aquatic environment, studies of degradation rates must be made over a range of conditions using

aqueous media which approximate natural conditions.

TCIN is known to undergo microbial degradation in soil to its 4-hydroxy phenolic derivative, DAC3701. The half-life of this degradative process is reported to be in the order of 2½-3 months in normal temperate soils (Stallard and Wolfe, 1967). DAC3701 is more soluble than TCIN, and would be more likely to travel into the environmental water compartment at a greater rate than TCIN. It may, therefore, present a toxic hazard to aquatic organisms. DAC3701 is also known to be produced by aqueous hydrolysis of TCIN, albeit slowly (Szalkowski and Stallard, 1976). TCIN is known to readily undergo photochemical reactions with organic solvents, and it is highly probable that this behaviour would extend itself to a propensity for environmental photolysis (Satoh, 1979). Volatilization may also be a potent force in the environmental dynamics of TCIN. TCIN is readily put into the vapour phase at low pressure, and is sublimatory at and below its melting point of 250 - 251° C.

In contrast, Chowdhury et al. (1981) found that 2,6-dichlorobenzonitrile (dichlobenil) was metabolized by only 17% when incubated with soil over 61 days, 7% being the benzamide, the rest unidentified. Volatilization and mineralization of dichlobenil was minimal over the 61 days.

A series of experiments were performed using TCIN and C^{14}-TCIN, in aqueous solutions from different sources, at different temperatures and with different substrates in order to investigate the biodegradative behaviour of TCIN in the aquatic environment.

2.2.2 MATERIALS AND METHODS

Chemicals

TCIN was purified by xylene extraction of technical grade Bravo (R) (Diamond Shamrock Corp.). After solvent removal at reduced pressure, the crude crystalline mass was recrystallized in acetone-water, and dried in vacuo. C^{14}- TCIN was prepared as described in Chapter 5, ⩾ 98% radiochemical purity, 0.734 mCi/mmol. Acetone was A.R. grade, and hexane was redistilled.

General test procedure

Aerated aquaria of water (20 l) were inoculated with an acetone

stock of TCIN or C^{14}- TCIN. At various time intervals, 240 ml samples were extracted as described in 2.1.2. The hexane fractions were analysed by G.C. for TCIN (2.1.2), or radioanalysis for C^{14}- TCIN by counting 1 ml in 5 mls Dimilume - 30 on a Packard Prias-PL Tricarb scintillation counter.

Water was collected from the North West Bay River at two sites (Fig. 2.5). N.W. Bay River is typical of a small coastal stream arising in high woodland country, and serving a small agricultural area in its lower reaches. Site 1 was used to provide water typical of the non-agricultural upper reaches. Site 2 was used to provide water typical of the coastal agricultural zone of the stream. Rock substrates, with and without attached algae, were collected at site 2 in the same river.

Experiment 1

TCIN degradation over 10 days, 15° C

Experiments were run as above with the following conditions:

$[TCIN]_o$ = 20 ug/l; water = site 2

Tanks 1,2 = still water.
3,4 = aerated water.
5,6 = aerated water, with rock and aufwuchs substrate.

Analysis by extraction and G.C.. Sediment, collected by filtering water, and aufwuchs were taken at the end of the experiment from all tanks and suspended in acetone (5 ml). After stirring for four hours, 20 ml water was added. The mixtures were extracted with 5 ml hexane and the hexane fraction analysed by G.C. (2.1.2).

Experiment 2

TCIN degradation over 74 h, 5 and 15° C

$[TCIN]_o$ = 20 ug/l; water = site 2

Tanks 1,2 = aerated water with rock and aufwuchs substrate, 15° C.

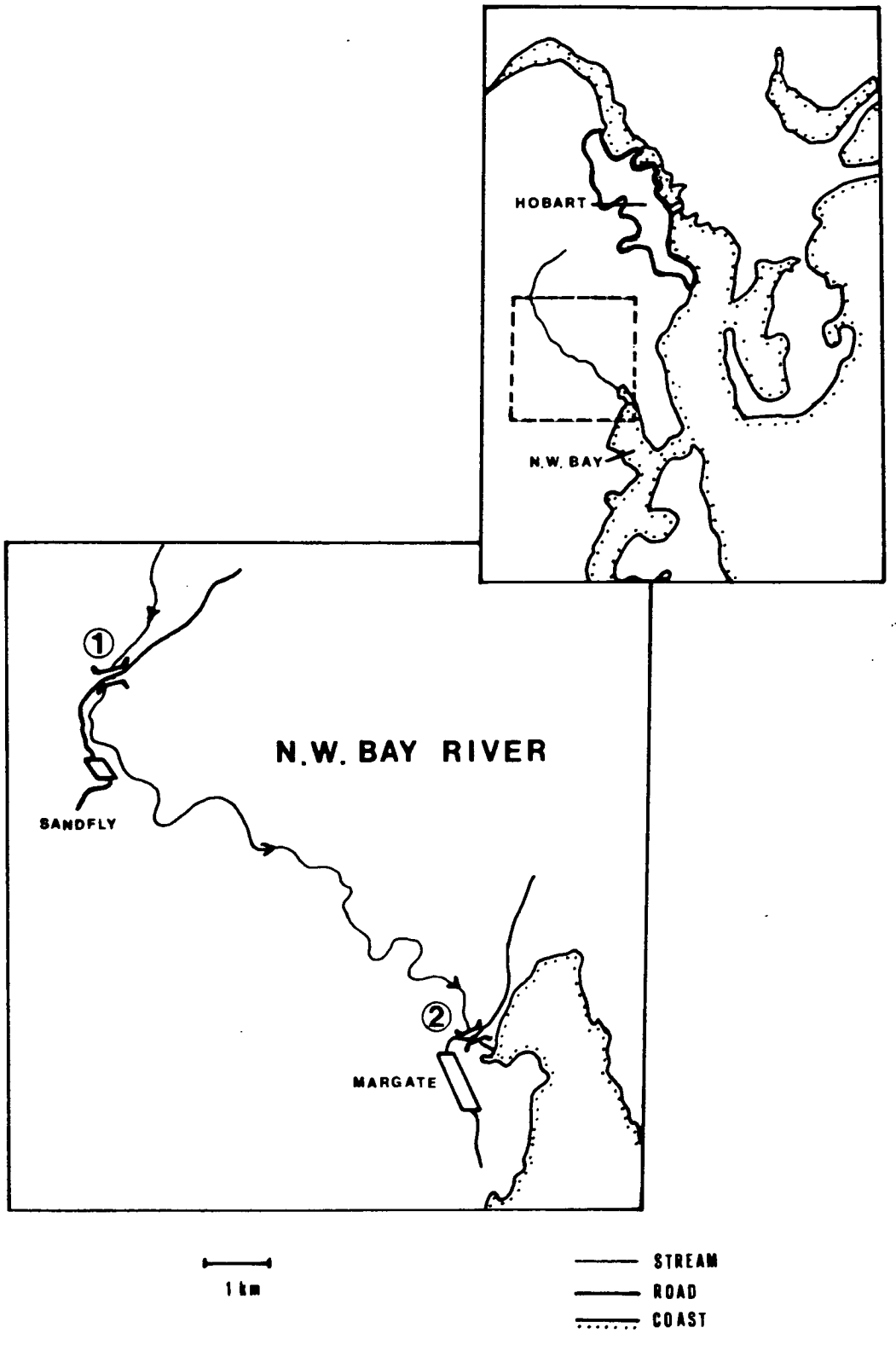


Fig. 2.5 Map of N.W. Bay River.
1,2 = Bridges where water was collected for biodegradation studies.

3 = still water only, 15° C.

4,5 = aerated water with rock and aufwuchs substrate 5° C.

6 = still water only, 5° C.

Analysis by extraction and G.C.

Experiment 3

TCIN degradation over 14 days: effect of substrate, water type and fish

$[C^{14}$- TCIN$]_o$ = 18 ug/l; temperature = 15° C; all tanks aerated.

Tanks 1,2 = site 2 water, rock and aufwuchs.

3,4 = site 2 water only.

5,6 = site 2 water, rock only.

7,8 = site 2 water, 2 Galaxias auratus (10 g each tank).

9,10 = site 1 water only.

11,12 = distilled water only.

Tanks were inoculated with 0.61 ml C^{14}- TCIN acetone stock to give an inital concentration of 18 ug/l and activity of 10^5 dpm/ml. Analysis was by extraction and radiocounting hexane and water fractions. Fish tissue sediment and algal aufwuchs were collected, dried, weighed and digested in Soluene (R) prior to radioanalysis, at the end of the experiment.

2.2.3 RESULTS

TCIN degradation curves from experiment 1 are shown in Fig. 2.6. Time-constants and half-lives of TCIN degradation are shown in Table 2.5, 2.6 and 2.7 for experiments 1, 2 and 3 respectively. Time-constants and half-lives for production of aqueous degradation products from TCIN, calculated from the aqueous counts after extraction, from experiment 3 are shown in Table 2.8. TCIN levels in algae and the fish from experiment 3 are shown in Table 2.9.

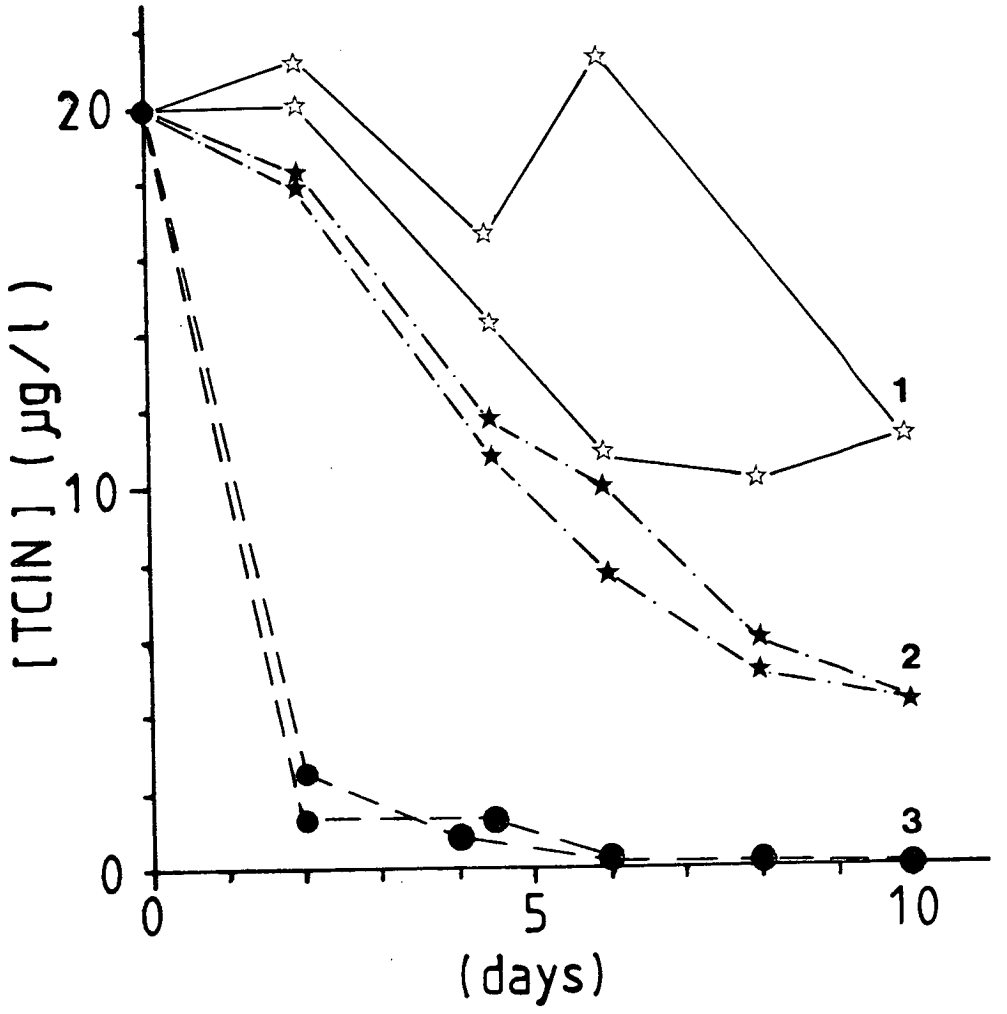


Fig. 2.6 Decrease of TCIN concentration in stream water at 15° C.

1 : unaerated water.
2 : aerated water.
3 : aerated water with circulation over rock substrate.

Table 2.5 Biodegradation parameters of TCIN, experiment 1

Conditions	Temperature (° C)	Half-life (days)	% remaining at 10 days
Still water	15	10.2	53
Aerated water	15	5.6	10.5
Aerated water, rock-aufwuchs substrate	15	1.1	0.0

Table 2.6 Biodegradation parameters of TCIN, experiment 2

Conditions	Temperature (° C)	Half-life (h)	% remaining at 75 h
Still water	5	150	75
	15	80	58
Aerated water,	5	13.9	4
rock-aufwuchs	15	7.7	≤ 1

Table 2.7 Biodegradation parameters of TCIN, experiment 3
Decrease in TCIN concentration. T = 15° C

Conditions	Water site	Half-life (h)	Constant b ($x10^{-2}$)	% remaining at 14 days
Distilled aerated water	-	106.3	-0.652 *	6.5
Aerated water	2	101.3	-0.684 *	5.7
Aerated water	3	107.3	-0.646 *	6.2
Aerated water, G. auratus	2	4.3	-47.9 **	4.4
Aerated water, rock-aufwuchs substrate	2	4.4	-46.7 **	4.3
Aerated water, rock surface	2	90.6	-0.765 *	5.0

* = regression of the form $y = a.e^{bt}$, t in hours, y in ug/l
** = regression of the form $y = a.t^{b}$, t in hours, y in ug/l
$N \geqslant 7$, $p < 0.05$ for all regressions. Rock surface area = 1000±200 cm^2 per aquarium

Table 2.8 Biodegradation parameters of TCIN, experiment 3
Increase in polar metabolite concentration. T = 15° C

Conditions	Water site	Half-life (h)	Constant b ($x10^{-2}$)	% present at 14 days
Distilled aerated water	-	567.3	-0.131	84.1
Aerated water	2	172.1	-0.406	85.2
Aerated water	3	161.3	-0.431	80.2
Aerated water, G. auratus	2	55.9	-1.240	90.1
Aerated water, rock-aufwuchs substrate	2	237.4	-0.292	81.5
Aerated water, rock surface	2	163.9	-0.423	91.4

regressions are of the form $y = 25 - a.e^{bt}$, t in hours, y in ug/l TCIN equivalents. $N \geqslant 7$, $p < 0.05$ for all regressions. Rock surface area = 1000±200 cm^2 per aquarium

Table 2.9 C^{14}-TCIN residues accumulated in aufwuchs algae and fish tissue over 14 days, biodegradation experiment 3

	Aufwuchs density	ug/g C^{14}-TCIN wet wt	dry wt	% of total tank dose	BCF (wet wt)
Aufwuchs	36.2 - 41.5 mg dry wt/100cm^2 rock surface	5.41	27.05	9.5	271
Fish tissue	-	0.35	2.5	3.4	18

where BCF = bioconcentration factor based on total residue equivalents and an initial dosing concentration of 20 ug/l

2.2.4 DISCUSSION

Degradation of TCIN was slow in still water, with 53% remaining at 10 days. Aeration caused an increase in degradation rate so that 50% of the TCIN was removed from the water at 5.6 days. This may be caused by enhanced volatilization, surface stripping and bacterial metabolism. The addition of a rock substrate with algal aufwuchs caused a far greater enhancement of TCIN degradation so that only 10% remained at 2 days, and none was detected at 10 days (Fig. 2.6).

Degradation of TCIN at 5° C was markedly slower than at 15° C. A comparison of degradative rates, in the presence of substrate, gives a rate-ratio value, or Q_{10}, of 1.8, in agreement with values expected for biochemical processes, with typical enzymatic activation energies around 10 kcal/mole (Larson et al., 1981). The half-lives of TCIN under these conditions were approximately 13 and 5 h for 5 and 15° C respectively.

Despite the fact that no significant differences in TCIN disappearance rates occurred between the three water types, the rate of appearance of TCIN water-soluble metabolites was significantly affected, increasing in the order:

distilled water		water 2		water 3
1	:	3.30	:	3.52

This indicates that the rate of generation of TCIN polar metabolites is influenced by microfloral and/or solute concentrations in the water.

The removal of the aufwuchs layer from boulders of roughly the same surface area as those left intact caused a dramatic decrease in the rate of TCIN disappearance from water, by a factor of 61 times. The rate of TCIN degradation was not significantly enhanced by the presence of the sterile doleritic rock surface in the aquaria. This showed that the aufwuchs layer played by far the dominant role in TCIN removal from the water column. Analysis of the aufwuchs algae showed that only 9.5% of the TCIN dose was incorporated into the algae, despite the fact that this represented a bioconcentration factor of 280 times. Benthic algae have been found to concentrate the highly chlorinated dieldrin by up to 30000 times in laboratory streams contaminated at 0.05 - 7 ug/l over four months (Rose and McIntire, 1970).

It, therefore, appears that stream-bed algae increase the rate of TCIN removal by enhancing the conversion rate to polar metabolites. In lotic waters, the ability of the aufwuchs to bioconcentrate TCIN, and to "strip" it from the water, may be more important than its apparent ability to metabolize it, due to the unequilibrated state of stream flow. The initial rates of algal TCIN degradation were estimated at 2.7 and 4.0 ug/h/g wet weight algae, or 56.5 and 72.0 ug/h/m^2 rock surface, from a 20 ug/l solution.

The addition of 10 g Galaxias auratus to TCIN solution increased the TCIN degradation rate by 25 times, and the rate of polar metabolite production by three times. It therefore appears that fish can readily take up TCIN from water, and metabolize it. The difference in relative rates also indicates a substantial TCIN accumulation. Initial uptake rates by fish were 0.73 and 0.81 ug/h/g fish tissue, 4 - 5 times lower than those for benthic algae. This difference may be related to the relative ease of diffusive access of solutes to the filamentous algal cells, whereas the primary route of fish uptake is restricted to one site, the gills.

Despite the highly chlorinated structure of TCIN, it is readily biodegraded in stream water, in contrast to other chlorinated hydrocarbons such as kepone and DDT (Chakrabarty, 1982). This may be due to the presence of the nitrile functions which cause the 4 and 6 chlorines to be more readily attacked and replaced by nucleophiles, facilitating hydroxylation. Insertion of chlorine on the aromatic nucleus of a hydrocarbon slows the rate of biological attack by electronegative deactivation (Chakrabarty, 1982), and it has been shown to inhibit the primary oxidation of chlorobiphenyls by microorganisms. In the case of TCIN, such oxidative processes are not possible due to the complete substitution of the aromatic nucleus. Direct attack is only possible by single nucleophilic species, such as hydroxyl and sulfhydryl groups. It is unlikely that straight dechlorination would occur. Indeed, Chakrabarty (1981) suggests that in microorganisms, "plasmids encoding dechlorination steps have not yet evolved in nature".

Three pieces of evidence suggest that TCIN degradation is regulated by microorganisms in stream water. First, the rate of degradation is faster than the rate of hydrolysis observed by

Szalkowski and Stallard (1976) in distilled water-buffer solutions. Secondly, the order of biodegradation rates in the different waters tested here suggests an increase in rate with a likely increase in microorganism density, although the latter was not measured. Thirdly, the Q_{10} value supports an enzymatic process. What the degradative processes are is unclear at present, although TCIN is converted to polar form(s). Considering previous work on soils, it is likely that the chief degradative product is the polar 4-hydroxy trichlorophthalonitrile (DAC3701).

In conclusion, TCIN was degraded slowly in river water with a half-life of 10 days. Aeration increased the rate of disappearance (half-life of 5 days), by a combination of volatilization and increased microbial activity. TCIN was apparently degraded by microorganisms in river water with a Q_{10} of 1.8 and the degradation rate increased downstream in a small stream. Rock substrate did not increase the rate of disappearance. Algal aufwuchs increased the disappearance rate by 61 times, with a half life of 5 and 13 h at 15 and 5° C, respectively, by conversion of TCIN to polar forms. Algae had bioconcentrated TCIN by 280 times by 14 days. Aufwuchs of substrates is expected to have a marked effect on the behaviour of TCIN in streams. Fish appear to take up TCIN readily, although at a lower rate per g than algae. They also metabolize TCIN readily, converting it to polar forms. The polar products of TCIN biodegradation were not identified.

TCIN is readily biodegraded at low concentrations (20 ug/l) and cannot be regarded as a persistant pollutant. It is unlikely that biodegradation will play a major role in the fate of TCIN in fast flowing streams, where volatilization and stripping by adsorption are likely to be dominant factors.

2.3 STREAM DYNAMICS OF TCIN

2.3.1 INTRODUCTION

Materials released into a stream at a point location disperse downstream, giving a time-concentration profile at any downstream point whose differential form,

$$Vi. \frac{dci}{dt} = Q_i (c_{i-1} - c_i) \quad \text{——} \quad \text{Eqn. 2.1}$$

(where c_i = concentration in the ith reach, of volume Vi where the flow rate is Q_i), cannot be solved without resort to involved computation and the use of empirically defined parameters for each particular case (Whitehead, 1980). This problem is made more complicated by the element of random noise in the input (e.g. variability in level, location and times of pesticide release into streams from ground/surface waters) and in the output (e.g. variability in stream flow and extent of stream degradation and stripping of pesticide materials) (Shahane, 1976).

Unless a stream is regarded as important enough to be investigated in detail, there is little point in performing a study of dispersion profiles when little information is available on patterns of pesticide release into the stream waters. Models of pesticide release events are influenced by such a wide variety of highly variable parameters that predictive estimates of stream pesticide concentrations are unreliable (Haith, 1980; Wauchope and Leonard, 1980), and empirical measures are easier to obtain and more accurate (McKimm and and Hopmans, 1978; Pearce et al., 1979).

Since the TCIN sprayed sites in the Brid River and Muddy Creek catchments were 15 - 20 km upstream from the trout-farms, it was felt that some indication of the behaviour of TCIN in these streams would be of value. The biodegradation study showed that stream bed materials with associated aufwuchs greatly increased the rate of TCIN removal from the water. In a small, longitudinally homogenous stream the parameters causing pesticide removal from stream water can be considered as constant per unit stream length. Release of a low concentration spike of pesticide into a stream and monitoring the downstream concentration should enable one to calculate a "stripping factor" which may be used in order to estimate the likely concentration bounds of a downstream reach. Such an experiment is described in this section. The suspended sediment water partition coefficient of TCIN was determined, in order to assess the role of sediment in TCIN stream dynamics.

2.3.2 MATERIALS AND METHODS

A single dosing experiment was performed on 10/2/1982.

Field Site

A small stream, Tucker's rivulet, was selected for the stream dosing study (Fig. 2.7). The flow, volume, length, geology and surrounding flora were all similar to that of the Brid and Muddy catchments and streams. Tucker's rivulet is a small tributary of the Great Forester River, and its catchment is adjacent and parrallel to that of Muddy Creek (Fig. 2.1). A uniform section of stream, 0.67 km long, without dams or weirs, and serviced by road bridges at both ends, was selected. The following parameters were measured or estimated on 10/2/1982 with the use of an Ottmeter current speed measuring device:

Flow = 0.052 m^3/s; length = 670 m; mean cross-sectional area = 0.27 $\pm$ 0.05 m^2 (N = 6); estimate of stream stretch volume = 181 m^3; measured stream current speed = 0.424 $\pm$ 0.071 m/s (N = 8); water temperature = 18° C.

Dosing

An acetone-TCIN-Rhodamine B stock was prepared (5.1 l : 77 g : 8.1 g) and released at 256-272 ml/min over 20 min by siphoning into the stream at the upper bridge. This stock was estimated to give maximum concentrations in the stream-stretch of 50 - 100 ug/l for TCIN, and 100 ug/l for Rhodamine B. This was not deemed harmful due to the rapid travel time and dispersion of TCIN in the stream.

Sampling and Analysis

Sampling was commenced at the lower bridge 4 min after dosing started. 250 ml samples were collected mid-stream every 3 min in acetone rinsed polyethylene bottles, and frozen for storage within 1 h. 240 ml of each second sample was extracted with 10 ml redistilled hexane by magnetic stirring for 20 min (efficiency of sampling and analysis = 96% by internal standard). Levels of Rhodamine B were found to be too low for fluorescence spectroscopy analysis.

Sediment partition coefficient

A number of samples were filtered through dried (100° C), pre-

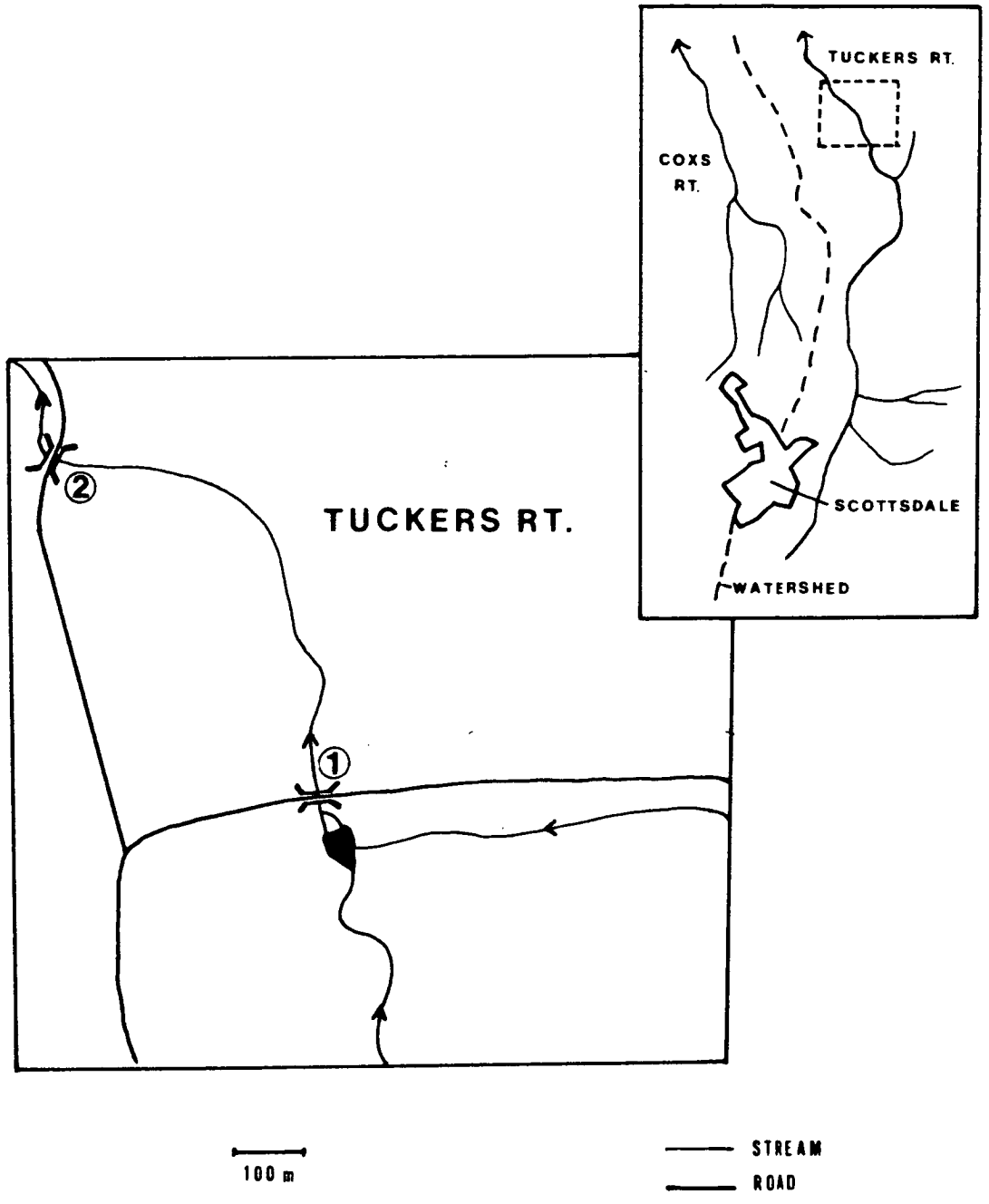


Fig. 2.7 Map of Tuckers Rivulet.

1 = Dosing site.

2 = Sampling site.

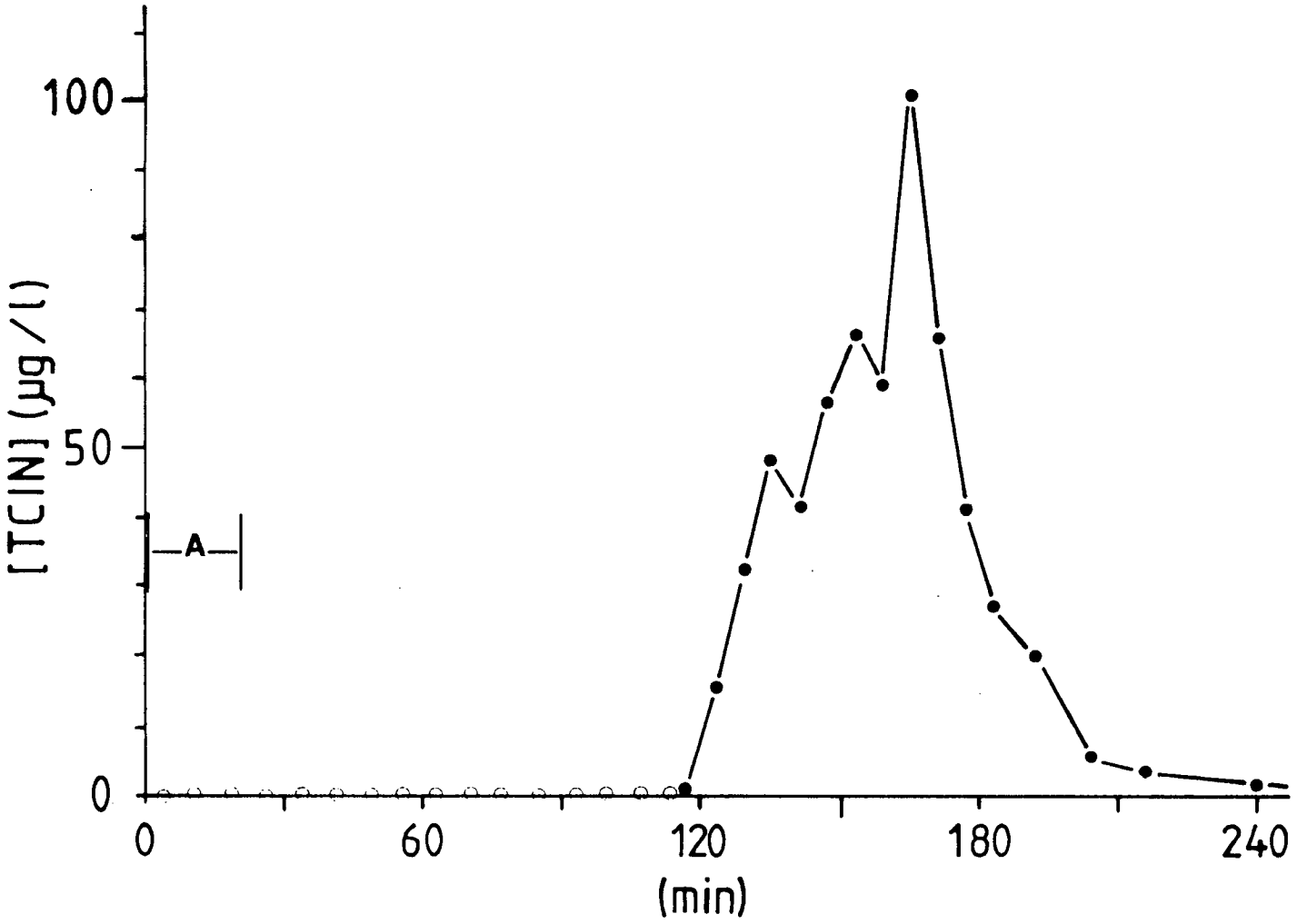


Fig. 2.8 TCIN concentration profile at sampling site 2 in Tuckers Rivulet, during the stream-dosing experiment. A = period of TCIN dosing at site 1. O = No TCIN detected; ● = TCIN detected.

weighed Sartorius glass filters. The filters were dried (60^{o} C), weighed and the upper-surface extracted (60 min) with 5 ml redistilled MeCN. The water was extracted as above. The hexane fractions were analyzed by G.C. for TCIN.

2.3.3 RESULTS

A plot of concentration against time after dosing commenced is shown in Fig. 2.8. Integration of the curve gives an estimate of 25.46 g TCIN passing the lower bridge.

This gives a Stripping Factor = $\frac{Ml}{Mu}$ = 0.0205/km,

(where Ml, Mu = total pesticide mass at lower and upper reaches respectively).

The peak concentration of TCIN was 100 ug/l, in agreement with the previous estimate. The half width of the peak at the lower bridge was 70 min compared with the 20 min dosing time, showing extensive dispersion. The travel time of the peak (front edge, 120 min) was much longer than expected, 27 min longer based on measured current speed, with the peak concentration passing the lower bridge at 165 min.

Analysis of suspended sediment in peak samples showed significant binding of TCIN to sediment (Table 2.10). The mean sediment-water partitioning coefficient = 4.95×10^{5}. Sediment-bound TCIN comprised a substantial proportion of TCIN in the samples.

2.3.4 DISCUSSION

The high stripping rate of TCIN by the stream materials indicates that most TCIN released in the upper reaches of small streams would be adsorbed or metabolized over a relatively short distance. Based on the stripping factor measured here, it would appear that if a release occurred in the upper reaches of such a stream at the water solubility limit of 600 ug/l for TCIN then, assuming constant flow, the concentration will drop to approximately

Table 2.10 Suspended sediment bound TCIN in TCIN-dosed stream water

Sample	N	Sediment load (mg/l)	TCIN concentration (ug/100 g)		% TCIN in sediment
			water (total)	sediment ($x10^6$)	
26	3	20.9	2.27	2.17	95.2
48	3	13.8	27.0	10.73	84.5
52	3	13.6	138.2	18.35	64.4

0.1 ng/l at 10 km downstream. Since TCIN releases are intermittant and low-level, it is highly unlikely that saturation of adsorption sites would occur. The stripping factor measured here is only applicable under conditions of similar flow and in similar streams, to those studied here. Multiple release times and sites, non-point releases and flow variations all require elaborate modelling (Whitehead et al., 1979), or some assessment of field disappearance constants (Stanley and Trial, 1980).

TCIN appears to be far more readily stripped from solution than Rhodamine (Smith, 1978). Rhodamine released into shallow lagoons in the A.C.T. showed a 43 to 57% loss over a period of 37 - 38 days. This can also be contrasted with the 5 h half-life of TCIN in 15° C aufwuchs-substrate biodegradation tests discussed earlier. Such differences in behaviour are related to differences in solubilities and reactivities (Chiou, 1981).

The extent of stripping of pesticide from stream-water is related to the organic matter - water partition coefficient. Chiou (1981) quotes several relationships established between the solubility and organic matter-water and organic carbon-water coefficients used in soil adsorption studies. Low solubility organic compounds are found to have high affinities for organic material and high lipophilicities.
The regression:

$$\text{Log } P_{ow-w} = -0.557.\log S + 4.040 \quad \text{——— Eqn 2.2}$$

where P_{om-w} = organic matter - water partition coefficient,

S = solubility (umol/l), r = 0.994 and N = 15.

predicts a value for TCIN of

$$\text{Log } P_{om-w} = 7.185.$$

The value obtained for the suspended sediment-water partition coefficient for TCIN is

$$\text{Log } P_{s-w} = 5.695.$$

Suspended sediment in Tuckers Rivulet was not all organic material. Several reaches of the bottom were of shifting sand, and filtered sediment contained numbers of micaceous flecks. It appears, despite this, that an appreciable amount of TCIN is associated with suspended

sediment, and will be unavailable for toxicological interaction with non-filtering aquatic biota.

A highly lipophilic organochlorine pesticide, Kepone, has a P_{s-w} value of 5×10^3 based on field studies in a contaminated river (Strobel et al., 1981). This value confirmed estimates determined in the laboratory. Kepone equilibration between sediment and water was rapid despite low total levels (0.3 - 10 ng/l). Elevated river discharges did not affect the partition coefficient. Thus the majority of Kepone loss from the watershed was by association with sediment. Similarly toxaphene, a chlorinated camphene, was found primarily associated with suspended sediment (93%) (McDonald et al., 1981). D.D.T at 0.17 ug/l in natural brown water was found to have a P_{s-w} of 1.58×10^4 and was primarily organic - colloidally associated (Poirrier et al., 1972).

Ben and Turner (1978) measured the rates at which changes in channel discharge were propagated downstream in small streams and calculated the relationship between discharge q (l/s) and the volume of water carried per unit channel length, h (l/m), as:

$$q = 0.00308.h^{1.81} \quad \text{——} \quad \text{Eqn. 2.3}$$

They found that this relationship adequately allowed for calculation of travel times along stream reaches. Using equation 2.3, a current speed of 0.241 m/s can be calculated for Tuckers Rivulet during the dosing experiment. The measured value of 0.424 m/s was based on Ottmeter measurements. It is not a measure of the overall stream speed as influenced by bottom friction, eddies and bends, since it was measured only mid-stream and at the stream sides at 8 locations. The measured speed of the TCIN peak (front edge) was 0.109 m/s, much lower than either of the above estimates. This discrepancy may be due to the fact that TCIN was primarily associated with sediment transported by the stream.

Nesbitt and Watson (1980 a,b) studied the degradation of 2,4-D in waters of a West Australian river. They correlated 2,4-D degradation with levels of sediment and dissolved organic matter, but not to microbial levels. It appears that the "degradation" of 2,4-D may actually be a decrease in concentration due to binding to suspended sediments. Equation 2.2 predicts a 2,4-D P_{s-w} value of 132,

and it is highly likely that 2,4-D transport is related to sediment binding as in the case of TCIN. Nesbitt and Watson did not suggest this possibility, and attributed the cause of degradation to sediments "providing an interface for bacteria - 2,4-D - nutrient interactions", despite the lack of correlation of 2,4-D degradation rates with microbial activity.

In conclusion, therefore, it appears that TCIN is rapidly removed from stream water by adsorption and metabolism by organic material and biota, with a stripping factor of 0.205/km at 18° C in Tucker's rivulet. TCIN is primarily associated with suspended sediment, up to 60-90%, with $\log P_{s-w}$ of 5.695. Equilibration between sediment and water is rapid. The stream transport rate of TCIN may be influenced by the rate of sediment transport. It appears that small streams can act as an effective 'filter' for TCIN at low concentrations. No indication was gained of TCIN - stream interactions over sustained periods of input.

CHAPTER 3

ACUTE TOXICOLOGY AND RESPIRATORY RESPONSES OF FISH TO CHLOROTHALONIL

3.1 ACUTE TOXICOLOGY

3.1.1 INTRODUCTION

In order to gain a relative toxicological standard by which to scale exposure experiments, and with which to set safety standards, an LC50 bioassay test must be conducted first. The LC50 is the concentration of a toxin which produces a 50% lethal response in a population. It is a function of time, standard times being 24, 48 and 96 hours. Standard procedures for the performance and interpretation of such tests using fish have been detailed by Sprague (1969, 1970).

An LC50 usually follows a standard form: a set number of fish is placed in each of a number of aquaria filled with water at each of a set of known toxin concentrations. The dead fish are counted at intervals up to 96 hours, with all loss of movement being taken as the point of death. In static tests, the water is either left unchanged or is changed periodically during a test. In both cases, changes in the concentration of toxin must be taken into consideration due to absorption, adsorption, hydrolysis and volatilization.

Flow-through tests are now generally considered mandatory for accurate LC50 testing, and, indeed, any exposure experiment where constancy of concentration is desired. There are a number of designs of systems which allow delivery of a constant stream of toxicant with serial dilution producing a range of concentrations in exposure aquaria (Mount and Brungs, 1967; Stark, 1967; Hodson, 1979). Rate of flow is generally such that the oxygen demands of the fish are met, and that the toxicant levels stay constant through each of the exposure tanks. In all cases, rigorous sampling of the water in the exposure tanks should be carried out for toxicant levels, and no reliance should be based upon expected concentrations. Adsorption of toxicants, especially lipophilic organic materials, is a pronounced phenomenon in glass and plastic containers (Sharom and Solomon, 1981), even in a flow-through system. For both static and flow-through

tests, fish should all be of a similar size class, from the same stock, and well acclimated to test conditions.

Even though most of the above details have been widely publicised, standard LC50 testing procedures are still not adhered to in many reported experiments in aquatic toxicology.

Once details of fish death at different times have been recorded, the LC50 values are estimated by probit estimation calculations, converting percentage death to probit values and computing the 50% value in either a log or normal scale. If LC50 values are calculated for a range of exposure times, an asymptotic relationship is developed whose asymptote is taken as a time-independent LC50 value for the toxin.

Unlike mammals, in fish there is generally only one dosage route employed, that is, exposure through the gills by free swimming in the medium. Dosage by intraperitoneal injection, gavage or through the diet is performed very rarely, and results from such tests cannot be directly related to responses to environmental levels.

LC50 data on TCIN is poor and variable (Table 3.1), and no toxicological data for any galaxiid species has been published. Indeed this study represents the first detailed toxicological study on the galaxiid genus.

Flow-through 96 h toxicity tests were performed with TCIN on the species: Salmo gairdneri, Galaxias maculatus, G. truttaceus and G. auratus. Salmo gairdneri was studied due to the suspected TCIN pollution in the Brid River and Muddy Creek catchments, both of which feed the trout-farms discussed earlier. It is also a well established standard test species in aquatic toxicology, and thus serves as a yardstick by which the relative toxicological susceptibility of the Galaxias genus can be assessed. The first two galaxiid species are common and abundant in low-lying coastal streams, likely to pass through agricultural land, in South and Eastern Australia. G. maculatus also occurs in New Zealand and Chile. Both of these species occur in the Brid River and Muddy Creek catchments. G. auratus is a locally abundant species restricted to two relatively uncontaminated lakes in the central highlands of Tasmania. It was tested for comparative purposes.

Flow-through tests with TCIN on S. gairdneri were also performed at low oxygen levels, to assess the effect of normal summer

Table 3.1 Previously published lethal toxicological data for TCIN in fish

Species	Temperature (° C)	Formulation	Term	Test type	Value (ug/l)	Source
S. gairdneri	-	TCIN	Estimated MTL	-	250	BCPC, 1979
Lepomis macrochirus	-	TCIN	Estimated MTL	-	390	BCPC, 1979
Ictalurus punctatus	-	TCIN	Estimated MTL	-	430	BCPC, 1979
"Roach"	9 - 15	Daconil	48 h LC100	static	100	Perevoznikov, 1977
Gambusia affinis	25	TPN	48 h TLm	-	90	Nishiuchi, 1977
"Killifish"	25	TPN	48 h TLm	-	90	Nishiuchi, 1977
Poecilia reticulata	25	TPN	48 h TLm	-	200	Nishiuchi, 1977
Cyprinus carpio	25	TPN	48 h TLm	-	250	Nishiuchi, 1977
Cyprinus carpio	25	TPN	48 h TLm	-	67	Nishiuchi, 1979

oxygen levels at the trout-farms suspected of being contaminated on the toxic response of S. gairdneri to TCIN, and with a co-exposure to acephate, to assess the likely effect of environmental co-exposure. An LC50 test with DAC3701 on S. gairdneri was also carried out to assess the relative toxicity of this environmental breakdown product of TCIN.

3.1.2 MATERIALS AND METHODS

A flow-through toxicant delivery system based on the design of Mount and Brungs (1967) was built to supply five 40 l tanks with a range of toxicant concentrations, and one control tank with uncontaminated water (Fig. 3.1). Calibration curves for the system and details of methods of useage are given in Appendix 2.

The delivery system consisted of a 200 l stock tank filled daily with water mixed, typically, with 5-10 ml toxicant-acetone stock solution, to give a final water stock concentration of 0.5 mg/l TCIN (83% of water solubility). This stock was transferred by peristaltic pump at 140 - 150 ml/min into one end of the baffle tank (Fig. 3.1, B). The baffle tank delivered TCIN concentrations ranging from 5 to 100% of the highest level to the five tanks by a serial dilution of approximately 50% per baffle section. This dilution could be altered by changing baffle tank inflows and outflows. Overall toxicant strength could be changed by changing peristaltic pump rate. The header tank (Fig. 3.1, C) received water from a primary constant-level header tank (Fig. 3.1, E), supplied with charcoal-filtered Kingborough tap-water. All tubing other than glass diluter delivery tubes, was of polyethylene. All tanks were glass.

The use of a carrier-solvent, acetone, was necessitated by the low solubility and solution rate of TCIN in water. TCIN solubility in water is 0.6 mg/l, and in acetone is 20 g/l. Acetone solutions stirred into water allowed rapid solution of TCIN at final concentrations < 0.6 mg/l. The use of carrier necessitated use of a second pump delivering acetone-water stock to the control tank, to give a final tank acetone concentration equal to that of the highest concentration exposure tank. These levels were always ≤ 10 mg/l, far below the published 24 h LC50 value of 6,100 mg/l of acetone to S. gairdneri (Majewski et al., 1978), and below levels known to affect physiological functions

(Klaverkamp, 1982). No mortality or behavioural symptoms of toxicosis were observed in controls.

Flow-rates in exposure tanks were maintained at around 15 l/h giving a 95% replacement time of 6.35 h, and 9 tank volume equivalents per day. This is well within guidelines set by Sprague(1969, 1970). Oxygen levels were routinely tested with a Yellow Springs International oxygen probe-meter, and by the azide modification of the Winkler titration (Standard Methods, 1975). TCIN was purified as described previously. DAC3701 was synthesized by the method of Heilman et al. (1978).

In order to establish the concentration range suitable for the flow-through tests, a range-finding static bioassay was first carried out. Ten S. gairdneri were placed in each of six 52 l polythene bags filled with water, attached to inflatable rings floating in a swimming pool. The water was aerated, and toxicant-acetone stock added to give final concentrations of 7.6, 76, 380 and 760 ug/l and two with acetone only. Acetone concentrations were 185 mg/l. On the basis of this experiment, TCIN concentration ranges for flow-through experiments were typically from 3 - 50 ug/l. DAC3701 concentrations were 20, 45, 60, 104 and 200 ug/l.

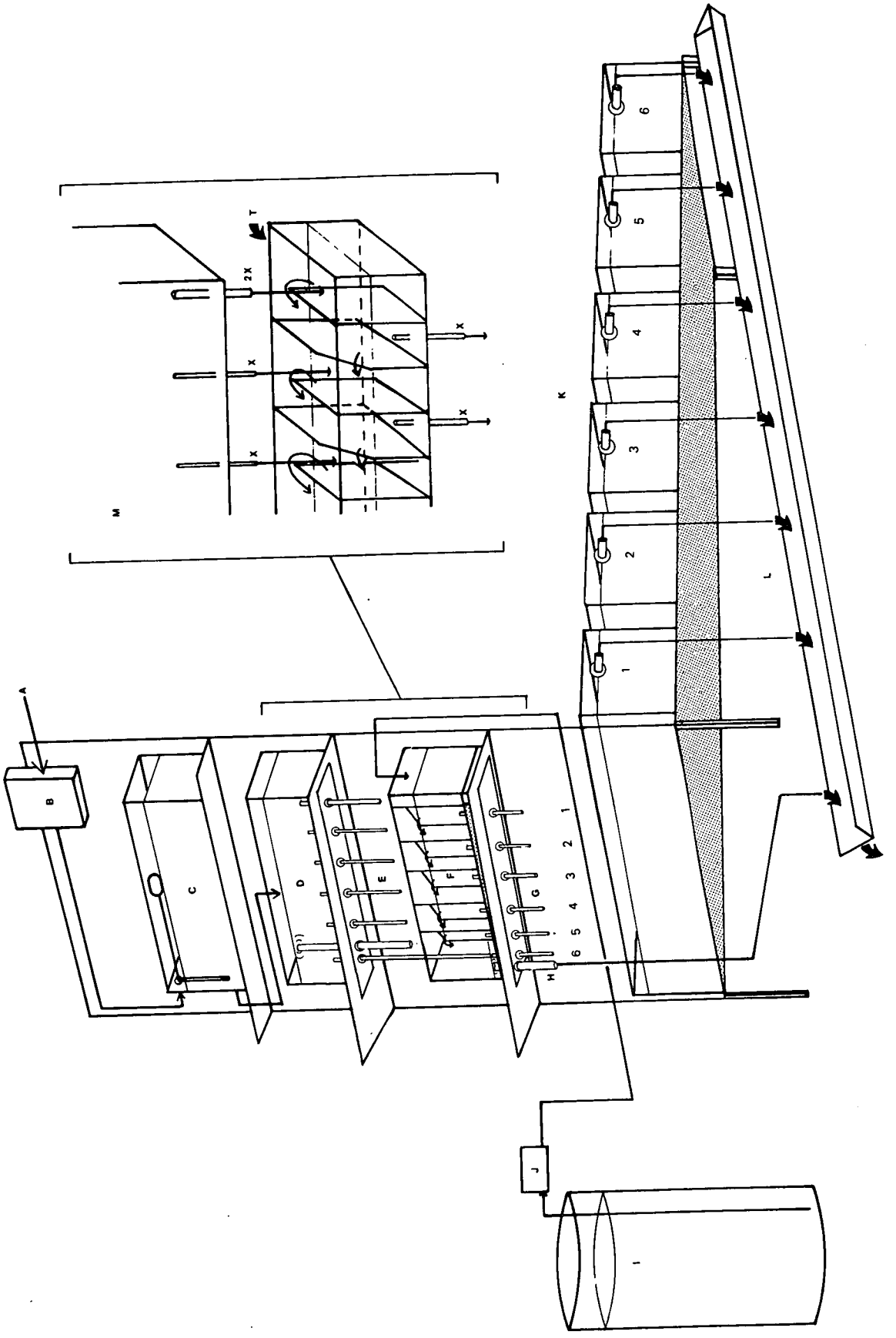
For the low-oxygen TCIN LC50 test, a frame was added above the header tank of the diluter system on which was supported a flow-through nitrogen-bubbling oxygen-stripping column (Fig. 3.2). Nitrogen gas (High purity, CIG) ascended through the glass marble filled column (Fig. 3.2, A) after leaving the diffuser stone, and discharged through a venting pipe (Fig. 3.2, B). Water from the charcoal-filter assembly entered the small aerated header tank (Fig. 3.2, C) and descended the gassed column. It then passed into the small side chamber (Fig. 3.2, D) and passed into the header tank of the toxicant dilutor system (Fig. 3.1, D). The dilutor system delivery pipes were extended to below the baffle tank water surface to avoid re-aeration.

Oxygen levels of the water leaving the stripping column and entering the exposure tanks were monitored by Winkler titration and nitrogen gas flow was set to give a constant water oxygen concentration of 4 - 5 mg/l. Tank concentrations were approximately 5 mg/l.

For the acephate co-exposure experiment, the maximum crop-spray concentration ratio of 1.23 : 1 (acephate : TCIN) was used. The

Fig. 3.1 Flow-through diluter dosing system used for LC50 and exposure experiments on fish in this study.

A = mains water inlet.
B = charcoal filter assembly.
C = primary constant-level header tank.
D = secondary delivery header tank.
E = diluter delivery tubes and overflow.
F = sloping diluter baffle tank.
G = toxicant delivery tubes serving tanks 1 - 6.
H = main overflow.
I = toxicant stock drum (200 l).
J = peristaltic pump for delivery of toxicant to baffle tank.
K = six 40 l covered exposure tanks. Inflow at far ends.
L = outflows.
M = detail of diluter system, with flow directions and relative values indicated. See Appendix 2 for details on the mode of operation.
T = toxicant entry point.


A
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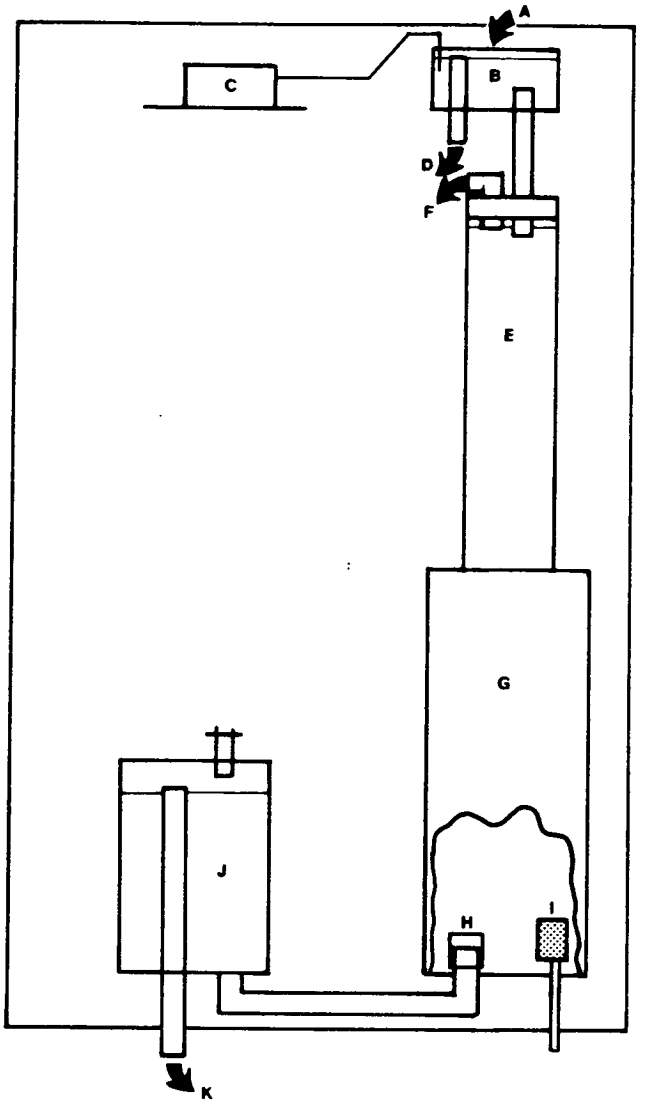


Fig. 3.2 Flow-through deoxygenation column.

A = Mains water inlet.
B = Constant level header tank.
C = Aerator.
D = Overflow.
E = Glass marble-filled column.
F = Gas vent.
G = Plastic marble-filled column.
H = Screened outflow pipe.
I = Diffuser stone nitrogen gas inlet.
J = Constant level distributor tank.
K = Deoxygenated water outflow.

required amount of Orthene was added directly to the water stock tank, dissolved in a little water.

Salmo gairdneri (6–11 g, mean wt. 9 g) were supplied by Sevrup Fisheries, Bridport, Tasmania. Galaxias maculatus (7–10 g)and G. truttaceus (8–20 g) were caught by electrofishing in Risdon Brook, Snug River and Browns River, S.E. Tasmania. G. auratus (7–11 g) was caught by electrofishing in Lake Sorell, Tasmania. The fish were bathed in 0.2% w/v methylene blue solution as an antifungal preventative. They were placed into the flow-through system, usually 15 to a tank, and fed once daily to satiation. S. gairdneri was fed with commercial dry pellet food (Mons and Affleck, 35–40% protein by Kjeldhal N-analysis); the other species were fed with chopped earth worms. All fish were collected in late 1981 and late 1982 and experiments were performed from November to January, when water temperatures were between 13 and 16° C.

The fish were maintained at least 10 days in the system prior to experiments; feeding was stopped 24 h prior to experiments. In the low-oxygen test, fish were acclimated to low oxygen for 4 days prior to the experiment. During an experiment, water samples were taken twice daily and analysed for TCIN by the method outlined in 2.1.2. Experiments were run for 96 h. Dead fish were counted and removed at various intervals. All fish were frozen for later analysis, at -18° C. The calculation of probit values from mortality data and estimation of LC50 values were performed using a probit programme (Appendix 3), on a Hewlett Packard HP 9825 A micro computer.

3.1.3 RESULTS

LC50 values were plotted against exposure time and are shown, with regressions, in Figs. 3.3 and 3.4. Asymptotic LC50 values were estimated and are given in Table 3.2 with the 24, 48 and 96 h LC50 data.

The mean oxygen concentration during the low-oxygen LC50 test was 5.1 $\pm$ 0.24 mg/l, or 53% saturation. This agrees well with mean summer trout-farm pond oxygen concentrations of 4–5 mg/l at Bridport. Results for this test and the acephate co-exposure experiment are given in Table 3.2.

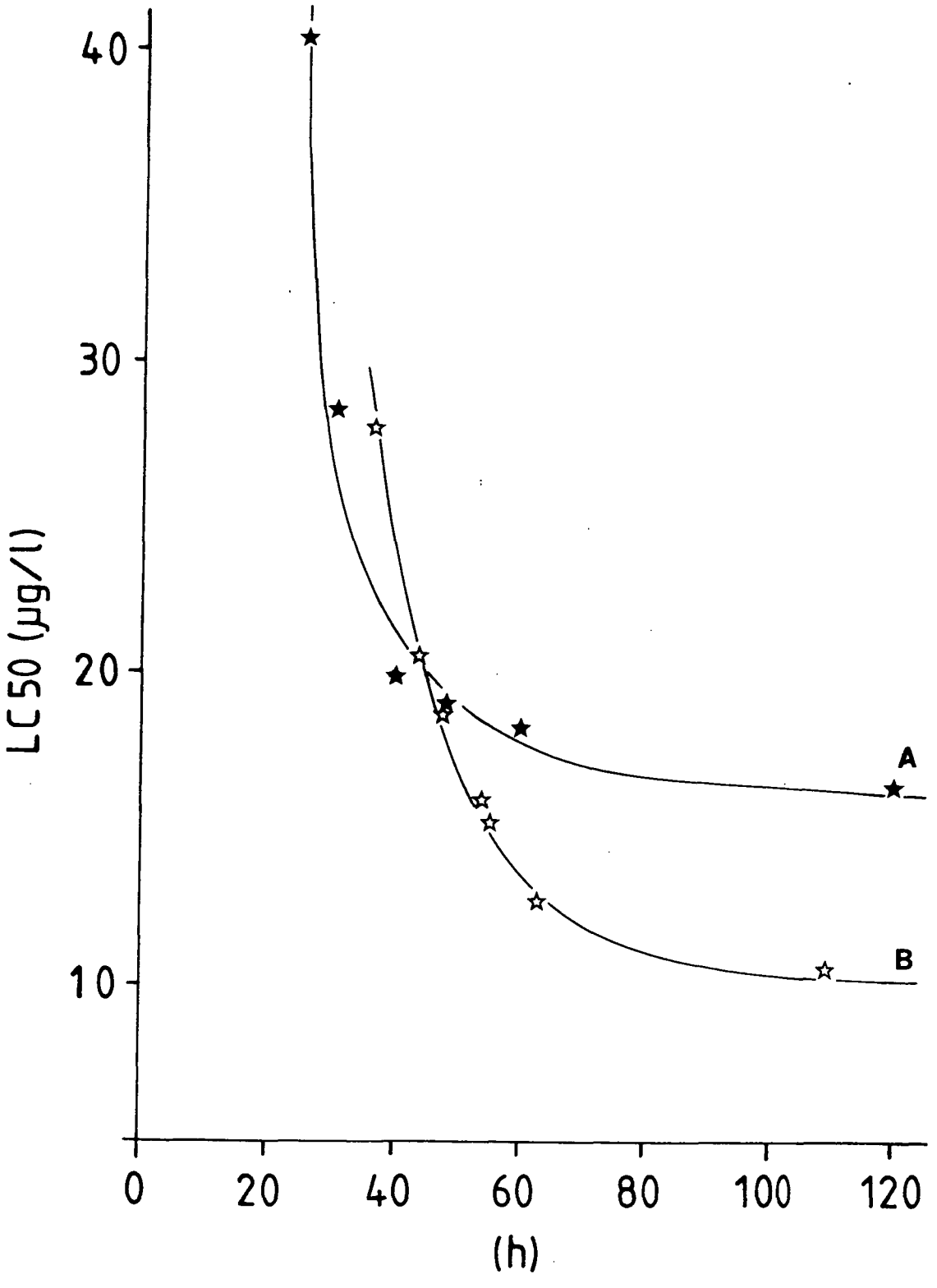


Fig. 3.3 TCIN LC50 versus time for S. gairdneri at 8.0 (A) and 5.1 (B) mg/l oxygen concentration.

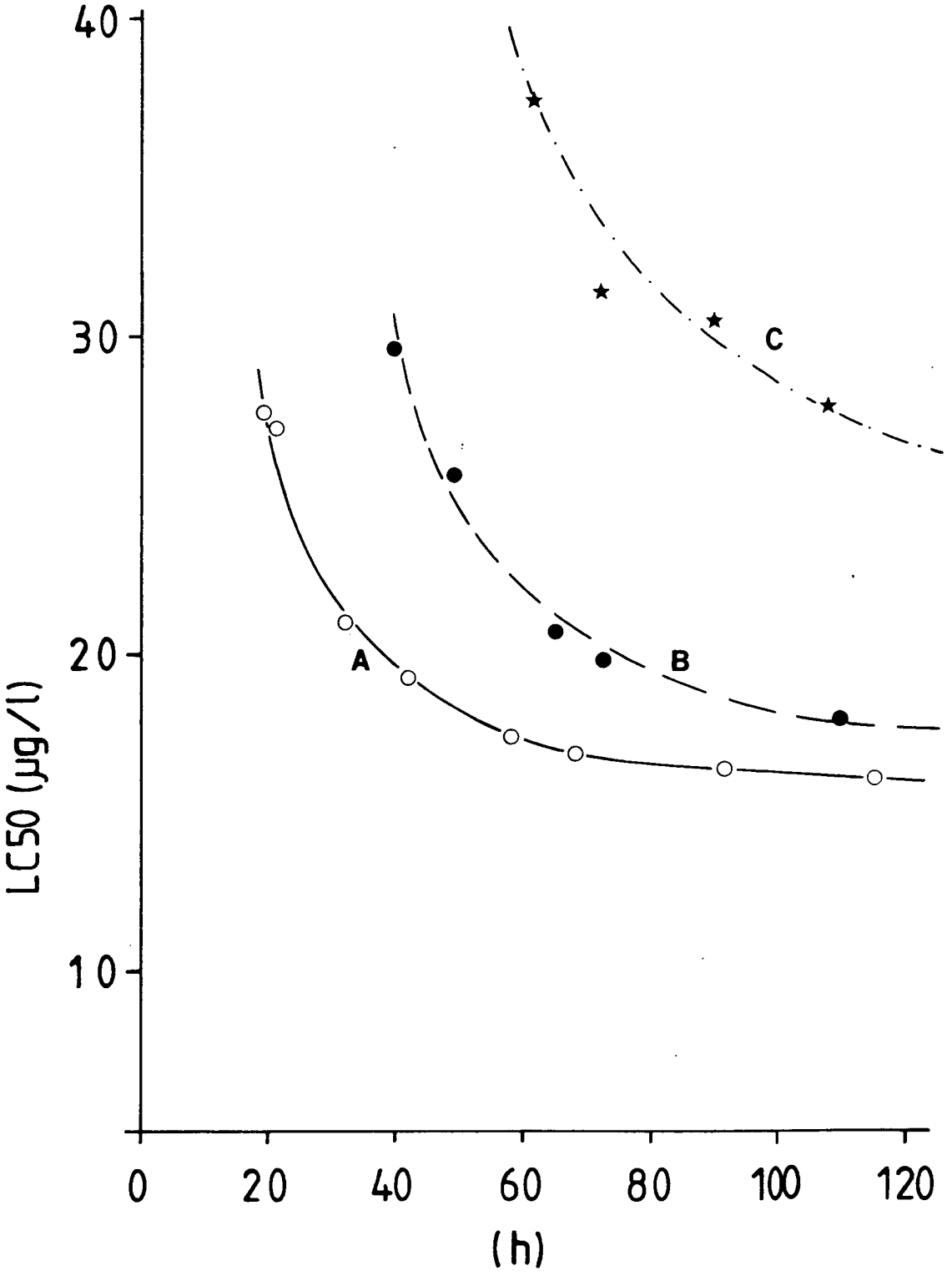


Fig. 3.4 TCIN LC50 versus time for G. maculatus (A), G. truttaceus (B) and G. auratus (C).

Table 3.2 TCIN LC50 data determined for *Salmo gairdneri* and *Galaxias* species

Species	Test type	DO (mg/l)	% sat.	T (°C)	LC50 (ug/l) 24 h	48 h	96 h	Asymptotic
S. gairdneri	Flow-through	8.0, 0.2	80	14	40.2	19.0	17.1	13.6
	Flow-through	5.1, 0.2	53	16	--	18.8	10.5	7.6
	Static	9.0	90	10	--	--	18.0	--
	Flow-through (acephate co-exposure)	8 - 9	80	15	--	17.1	14.3	12.2
G. maculatus	Flow-through	8 - 9	75	16	23.7	18.2	16.3	14.7
G. truttaceus	Flow-through	8 - 9	75	16	--	25.8	18.9	16.9
G. auratus	Flow-through	8 - 9	75	13	--	46.6	29.2	21.3

The 96 h LC50 values of the four species tested occurred in the order:

G. auratus > G. truttaceus $\overset{*}{>}$ S. gairdneri $\overset{*}{\geqslant}$ G. maculatus

where differences marked * were not statistically significantly different, based on the 95% confidence limits of probit estimates. The relationship:

G.auratus > S. gairdneri, G. maculatus

was significant at the 0.05 level.

The decrease of oxygen level from 80% to 53% saturation caused a 41% decrease in the 96 h LC50 value of TCIN to S. gairdneri. The difference is statistically significant at the 0.05 level.

Co-exposure to acephate caused a 16% drop in the 96 h LC50 value of TCIN to S. gairdneri but the difference was not statistically significant.

Exposure of S. gairdneri to DAC3701 of concentrations up to 200 ug/l for 6 days produced no mortality or signs of behavioural toxicosis.

3.1.4 DISCUSSION

All TCIN LC50 values are much lower than any of those previously reported by other authors (Table 3.1). Three reasons may be suggested for this disparity:

Firstly, the concentration of acetone may affect the toxic response to TCIN synergistically. The maximum level of 10 mg/l is far below the 24 h LC50 of 6,100 mg/l for S. gairdneri (Majewski et al. 1978), and below any level affecting physiological functions (Klaverkamp, 1982). No mortality or behavioural symptoms of toxicosis were observed in controls. A more subtle interaction cannot be ruled out, but it is unlikely. The static bioassay carried out for S. gairdneri gave a 96 h LC50 value not significantly different from the flow-through experiment result. Acetone levels in the static experiment were, however, much higher at 185 mg/l compared with $\leqslant$ 10 mg/l. Use of a solvent carrier is mandatory; details of carriers are not given in previous publications.

Secondly, the previous data may have been established with inadequate analysis of water TCIN levels during exposure, which did not take stripping into account. TCIN was found to be readily adsorbed onto surfaces during static and flow-through tests in this work, frequently giving concentrations as much as 90% lower than expected. It is probable that previous workers based concentration values merely on initial stock concentration figures. Thirdly, the disparity may be genuine, with some unknown cause.

On critically examining the sources quoted in Table 3.1, for published work on TCIN toxicity to fish, it was felt that a complete re-evaluation of this data is needed. The LC100 value quoted by Perevoznikov (1977) can be dismissed on the grounds that LC100 values are meaningless. A 100 per cent lethal concentration has no upper bounds, and can be obtained by concentration equal to or higher than the exact value required to kill 100 per cent within the time period tested. No details are given in Perevoznikov's paper of techniques and the low accuracy of the figure (0.1 mg/l) suggests a crude estimate.

The TLm values of Nishiuchi (1977), equivalent to the LC50, are also open to question. No indication of test techniques are given. The two results for C. carpio were apparently obtained under identical conditions. No explanation is put forward for the discrepancy between the two values which differ by 370%. No details on the Crop Protection Council figures were available from either the B.C.P.C. or the source of the data, Diamond Shamrock Cp., despite repeated requests.

On the basis of the results obtained here it appears that TCIN is much more toxic to fish than previously thought. Such low LC50 values are not, however, uncommon. In a recent review by Pickering et al. (1983), data presented on the LC50 values of 98 organic compounds included 20% less than 100 ug/l, 12% being less than 20 ug/l. In data presented on the LC50 values of 20 organic compounds to marine fish, 12 were less than 100 ug/l, and 7 less than 20 ug/l (Reish et al., 1983). Konemann et al. (1981) described a correlation between the water solubility of a toxicant and its toxicity to fish. By the regression:

$$\log 1/LC50 = -0.698.\log S + 0.09$$
$$(N = 27,\ r = -0.980),$$

one obtains an LC50 value of 382 ug/l for TCIN in fish.

Sabljic (1983) described the application of a molecular connectivity index to the prediction of LC50 values in fish. He recommends

the use of the zero-order molecular connectivity index 0_x where each non-hydrogen atom in a molecule is described by a value δ_i equal to the number of adjacent non-hydrogen atoms. 0_x is then calculated according to:

$$0_x = \sum_{i=1}^{n} (\delta_i)^{-0.5}$$

where n is the number of non-hydrogen atoms. It is an index of molecular topology, which by virtue of the recognised structure-activity relationships of molecules, can be used as a correlate in toxicological studies. Sabljic produced correlations between the 96 h LC50 and the no effect concentration values for sheepshead minnows using a wide range of 212 chlorinated benzene and alkyl compounds, many closely related in structure to TCIN. The 0_x value of TCIN is 10.88. Using the regressions of Sabjlic (1983), values of 77 and 32.9 ug/l are obtained for the 96 h LC50 and the "no effect" concentration respectively.

There are several methods which can be used to estimate a "safe level" of exposure for toxicants to fish (Sprague, 1971), all of which are subject to debate. Use of an application factor of 0.10 on the 96 h LC50 values gives safe levels far below the LC05 values estimated for the species tested here (Table 3.3). "No effect" concentrations depend on the parameters designated to indicate survival or undisturbed metabolic and/or reproductive function in the organism. Chronic effects of TCIN will be discussed in Chapter 4. When selected, on the basis of a lethal response and behavioural toxicosis symptoms, the highest "no effect" levels observed in these experiments fall between the other estimates.

The regressions of Konemann et al. (1981) and Sabljic (1983) do not produce agreements with the LC50 and "no effect" concentrations derived in this work. Fish species differences, and the fact that the regressions are based on solubility and molecular shape and not on the chemical characteristics of the toxicants, may well account for the absence of an exact prediction. However, values derived from Sabljic (1983) do fall within the correct order of magnitude, and consequently a structure - activity relationship based on benzenoid compounds related structurally to TCIN may go some way toward explaining the low toxic levels of TCIN.

Table 3.3 Safe levels of TCIN for freshwater fish species

Species	Safe levels (ug/l) Estimated on the basis of: Application factor	96 h LC_{05}	96 h "No effect"
S. gairdneri	1.7	15	8.7
G. maculatus	1.6	11.1	8.8
G. truttaceus	1.9	11.6	9.0
G. auratus	2.9	21.2	13.3

Table 3.4 Previously published data on the lethal toxicology of acephate toward S. gairdneri

Formulation	Term	Test type	Value (mg/l)	Source
Acephate	24 h LC50	Flow-through	2890	Klaverkamp, 1982
Acephate	96 h LC50	--	1000	BCPC, 1979
Tech. material (94%)	24 h LC50	Static	$\geqslant$ 50	U.S. Dept., 1980
Tech. material (94%)	96 h LC50	Static	1100	U.S. Dept., 1980
Soluble powder (75%)	96 h LC50	Static	730	U.S. Dept., 1980

The galaxiid species tested here have similar LC50 and "no effect" response levels to TCIN. G. maculatus is more susceptible than S. gairdneri, although only marginally and with exposures for less than 96 hours. Differences observed in toxic response may be due to differences in the way the pesticide is detoxified by the fish. This will be discussed in subsequent chapters. It seems, however, that as regards setting safety levels or limits on environmental aquatic residue concentrations of TCIN, data obtained from S. gairdneri, with an adequate safety factor, could safeguard the galaxiid species as well.

Low oxygen concentrations appear to act synergistically with TCIN toxicity in S. gairderi. The oxygen level of 5.1 mg/l used in the test is at the lower limit for growth and optimal metabolism in salmonids, but it is not itself harmful (Alabaster and Lloyd, 1982). Stress symptoms were not observed in control fish at this oxygen level.

Long and Siegel (1975) showed that TCIN can inhibit key respiratory enzymes. Such an effect on respiratory functions may explain the increase in sensitivity to TCIN with lowered oxygen and the loss of activity on TCIN exposure. Such a relationship indicates that the low oxygen levels experienced by fish at the Bridport trout-farm may enhance the toxic reaction to TCIN exposure. Since TCIN spraying is concomittant with low oxygen levels in the trout-farm, toxic reaction may well be instigated by levels of less than 10 ug/l on prolonged exposure.

The previously established values for acephate toxicity to S. gairdneri are shown in Table 3.4. They are all $\geqslant$ 700–1000 mg/l. Klaverkamp (1982) described significant changes in physiological parameters in S. gairdneri at the high level of 1,994 mg/l on 24 h exposure.

Acephate application rates range from 495–975 g active ingredient/ha. The ratio of 1.23:1 (acephate : TCIN) is the highest application rate ratio for the two compounds when sprayed together. This ratio was selected as the test ratio in the absence of information on environmental levels of acephate, noting that possible direct aerial application of the mixture to water courses could occur accidentally.

The small effect of acephate co-exposure on the toxicity of TCIN at the low acephate concentrations tested appears compatible with the low toxicity of acephate. The toxic action of TCIN is, therefore, by

far the dominant factor in environmental co-exposure.

Exposure of S. gairdneri to levels up to 200 ug/l of DAC3701, the phenolic derivative of TCIN, produced no lethal or behavioural response over 144 h. DAC3701 is, therefore, substantially less toxic to S. gairdneri than TCIN, and probably poses little environmental threat. The 24 h LC50 value of pentachlorophenol (PCP), a compound structurally similar to DAC3701, to Carrasius auratus is 270 ug/l (Kobayashi, 1979). Tetrachlorophenol (2,3,5,6) has a 24 h LC50 of 750 ug/l toward C. auratus (Kobayashi, 1979), and a 96 h LC50 toward Cyprinodon variegatus of 1,870 ug/l (Sabljic, 1983). As in PCP, its phenolic character probably allows for metabolism by phenolic conjugation reactions and rapid excretion (Kobayashi, 1979). Its toxicity is also probably pH dependent.

Rosanoff and Siegel (1982) reported that DAC3701 was more toxic and persistent in rats than TCIN (oral LD50 332 mg/kg c.f. > 10,000 mg/kg for TCIN). The reverse occurs in fish. This difference in effect on rats is probably a function of the route of administration of the toxicant. TCIN has a high affinity for organic adsorption and is much less soluble than DAC3701. It is, therefore, not readily absorbed from the gut and passes through extensively unchanged. Eighty eight percent of orally administered TCIN was excreted unchanged in the faeces (F.A.O., 1975). DAC3701 would be more readily taken up by the gut than TCIN, and consequently able to exert its toxic action at a lower oral dose. Since dosing in toxicity testing of fish occurs in the medium by gill exchange of already dissolved toxins, a true picture of the relative toxicities of the two compounds is obtained.

3.2 RESPIRATORY RESPONSES

3.2.1 INTRODUCTION

Rainbow trout are known to be sensitive to pollutants at levels that are sublethal. Sprague (1968) showed that trout avoid zinc concentrations as low as 5 ug/l, approximately 0.01 of the 7 day LC50 value. This sensitivity of response is shown by physiological as well as behavioural responses to pollutants (Morgan and Khun, 1974;

Carlson and Drummond, 1977; Majewski and Giles, 1981). Responses to organic pollutants are known to be sensitive to environmental parameters such as temperature (Heath, 1977), pH (Howrath and Sprague, 1978), and low oxygen levels (Herbert, 1962; Alabaster and Lloyd, 1982).

The respiratory system of fish shows a number of sensitive physical responses to toxicant exposure. These include changes in frequency of opercular of movement, changes in the rate of coughing - rapid flow reversal over the gills, and changes in buccal pressure. Each of these can be used as indicators of a toxic condition in water at levels much lower than a lethal response. The coughing reflex in rainbow trout has been described in detail by Hughes and Adeney (1977), and in bluegill (Lepomis macrochirus) by Carlson (1982), and has been used in a number of toxicant response studies (Schaumburg et al., 1967; Carlson and Drummond, 1973; Sellers et al., 1975; Bass and Heath, 1977).

The response that is studied in a majority of such papers is that of the change in ventilatory or opercular movement frequency. This is measured without manipulation or restraint of the fish in small electrode chambers, and has been used as the basis of a number of designs for monitoring fish-toxicant responses (Spoor et al., 1971; Morgan, 1975; Sparks et al., 1972; Morgan and Khun, 1974; Slooff, 1979; Gruber and Cairns, 1981; Cairns and Garton, 1982). A recent trend is to study several responses of the respiratory and cardio-vascular system simultaneously (Sellers et al., 1975; Travis and Heath, 1981), although this requires physical manipulation and restraint of the animal.

The response in ventilatory frequency is generally one of stress-induced upward trend in frequency values which shows a concentration dependence in magnitude, time of onset and duration of the response (eg. Morgan, 1975). There are a number of problems associated with its uses as an indicator response. The opercular frequency itself is often quixotic in nature. Apart from diurnal fluctuations in opercular rate, there is a large element of apparently random fluctuation in the rate of respiration (Sparks et al., 1972). Respiration is frequently punctuated by periods of slow or intense activity, complete cessation of activity and coughing. Careful use of control periods based on using test fish as their own control, or a

large enough number of control fish and long test periods can eliminate some of these fluctuations, but not all. The level of response is not readily transferable from one situation to another, and there is a surprising lack of uniformity of approach to both test procedures and subsequent data manipulation (cf. Cairns et al., 1982; Thompson et al., 1982).

The raw data is collected in the form of chart traces made over selected time periods during an experiment. Hand counting of the traces is commonly used, although laborious, or counts are made by a computer system (Gruber and Cairns, 1981). Once the data is tabulated against time, a variety of approaches have been used to analyze them, including correlation of response time and magnitude with concentration (Morgan, 1975) and analysis of variance of frequencies assessing minimum concentration of response for a standard time period of exposure based on general trends (Sloof, 1979). Thompson et al. (1982), suggested a uniform time-series approach to the analysis of ventilatory frequency which recognised the inter-dependent nature of the data from within an experiment.

An initial experiment was carried out in order to assess firstly whether Salmo gairdneri will show a respiratory response to TCIN exposure,and secondly whether this response is concentration dependent. Short term exposure experiments were carried out in order to get some idea of the threshold exposure concentrations at which responses are elicited within 2 h exposure. A data collection system was designed in order to facilitate frequency data counting. This is described in detail.

3.2.2 MATERIALS AND METHODS

High Level Exposure

Salmo gairdneri (mean weight 10 g), acclimated at 15° C, were introduced into the electrode cells (A) of a screened and shielded recycling exposure system shown in Fig. 3.5. After 24 - 48 h acclimation with flow-through of aerated water, with acetone carrier, ventilation frequencies were recorded on chart paper for 30 min. Flow

rates were sufficient to give 95% replacement in 5 min (80 - 100 ml/min). The fish were then exposed to the pesticide solution with the same acetone-carrier concentration (10 mg/l) for 5 min, during which time ventilation frequencies were recorded. Recording was continued for 25 min. Four fish were exposed at each of four concentrations : 25, 65, 200 and 310 ug/l.

Median Level Exposure

Trout were introduced into the same electrode-cell flow-through system as above, but with four cells running in parallel, each containing one fish. The ventilatory frequency waveforms were recorded during an initial control period. The fish were then exposed to four concentrations of TCIN in increasing order: 70, 90, 120 and 150 ug/l. The concentrations were increased serially with recordings of ventilation frequencies being made during the last 30 min of a 2 h exposure period at each concentration.

Low Level Exposure

An experiment was run as for the median level experiment, with TCIN concentrations in the order : 30, 40, 50 and 70 ug/l. Another experiment was run with TCIN concentrations increasing in the order: 10, 20, 30, 50,60 ug/l. Four fish were used in each experiment. Output from the cells was amplified and filtered (0.5 Hz lower bound) to reduce body movement signals.

Chart recorder traces were analyzed by hand-counting the observed peaks. In the median and low-level exposure experiments, average ventilation rates in beats/min were calculated from ten sample counts of the filtered output. Difference from control periods were tested for significance (Student's t-test). In the high-level exposure experiment, output was unfiltered and gross body movements were estimated on the basis of large amplitude, low-frequency peaks.

The water in these experiments was analyzed for TCIN concentration by hexane extraction and G.C. analysis (2.1.2).

Fig. 3.5 Recycling exposure system for respiratory-frequency response experiments using fish.

A = Fish in cylindrical flow-through cell. Two aluminium plate electrodes are set against the inside of the cell.

B = Outflow reservoir, aerated.

C = Pump, activated by constant level switch on D.

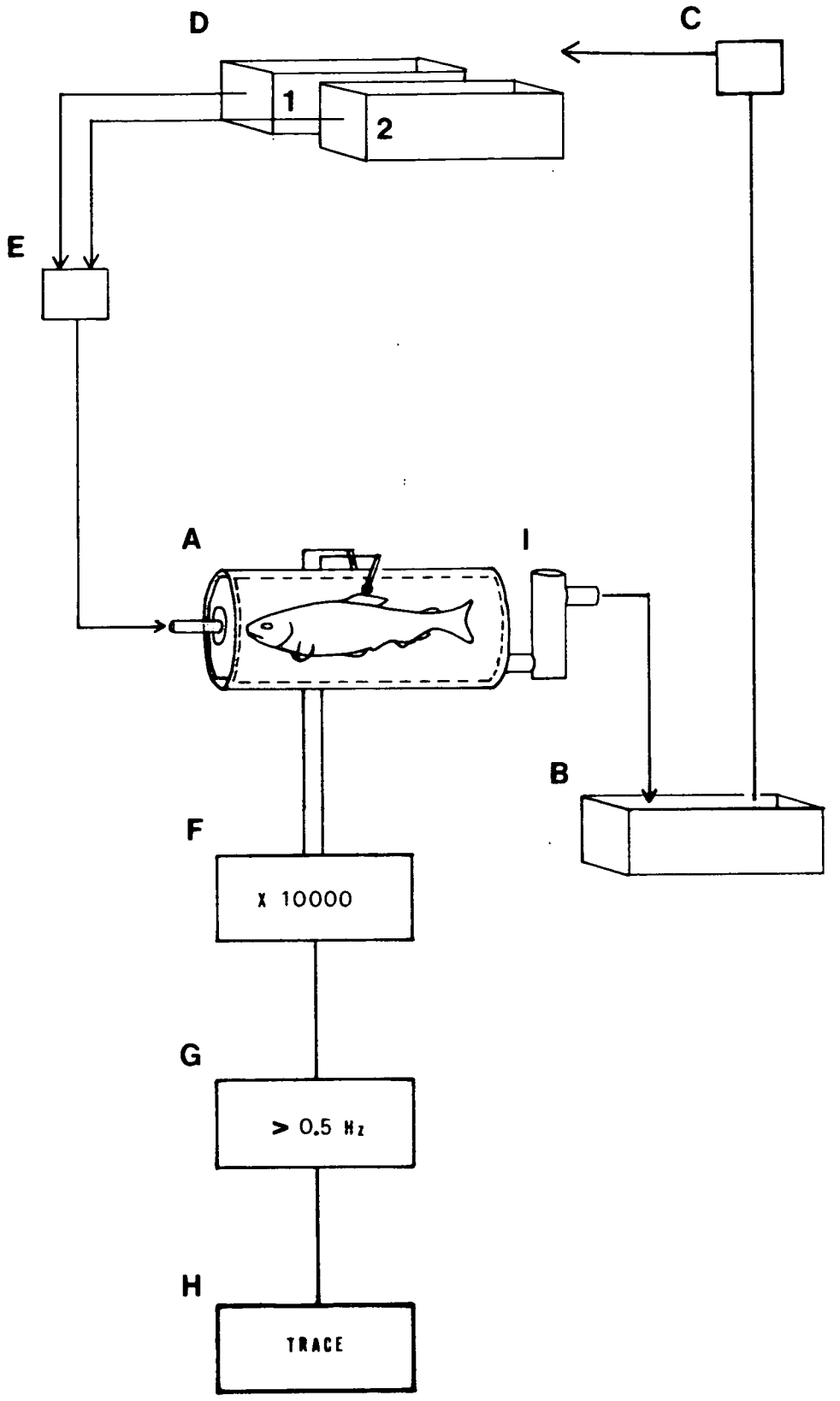
D = Constant level header tanks.
1: contains acetone-only control water.
2: contains pesticide contaminated water.

E = Solenoid switching valve.

F = Signal amplifier.

G = Low-frequency filter.

H = Outflow cell for oxygen probe.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C
1
2
E
A
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B
F
X 10000
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> 0.5 Hz
H
TRACE

3.2.3 RESULTS

The results for the high-level exposure experiment showed a dramatic increase in ventilatory frequency with concentration exposure (Fig. 3.6) during the 30 min response period. A typical response profile is shown in Fig 3.7. Gross body movements also increased dramatically on exposure to all concentrations, accompanied by bursts of very rapid ventilation, indicating acute stress (Fig. 3.8). The plateau in the response curve indicated that the response was maximal at 200 - 310 ug/l.

The results of the 2 h exposure period experiments are shown in Fig. 3.9, graphed as change in mean ventilation rate from the control period for each fish against concentration of TCIN. A marked concentration dependence both on the extent of response and the number of fish responding is demonstrated. A "threshold" level in which minimal response is shown for 2 h exposure can be estimated at 30 ug/l, 1.6 times the 96 h LC50, where one in eight fish responded. No significant response was shown by fish exposed to 10 and 20 ug/l TCIN for 2 h.

3.2.4 DISCUSSION

These preliminary experiments were designed to indicate the concentrations at which overt respiratory responses were shown by S. gairdneri within 2 h of TCIN exposure, and the extent and nature of the response. The trout-farm owner concerned with this project had indicated that rainbowtrout in the Bridport trout-farm showed acute respiratory stress within several hours of aerial spraying of the catchment with TCIN. The stream levels analyzed after spraying operations (2.1.2) do not appear to be high enough to cause such a reaction in the light of these experiments. Long-term exposure experiments would be justified in this context, but it is unlikely that TCIN levels of 1 - 5 ug/l could cause acute respiratory stress responses within a few hours of exposure. The experiments in this work were performed at saturated oxygen levels. Oxygen levels in the Bridport farm were about 50% saturation during summer. It is possible that a synergistic relationship exists between responses to TCIN and oxygen levels which

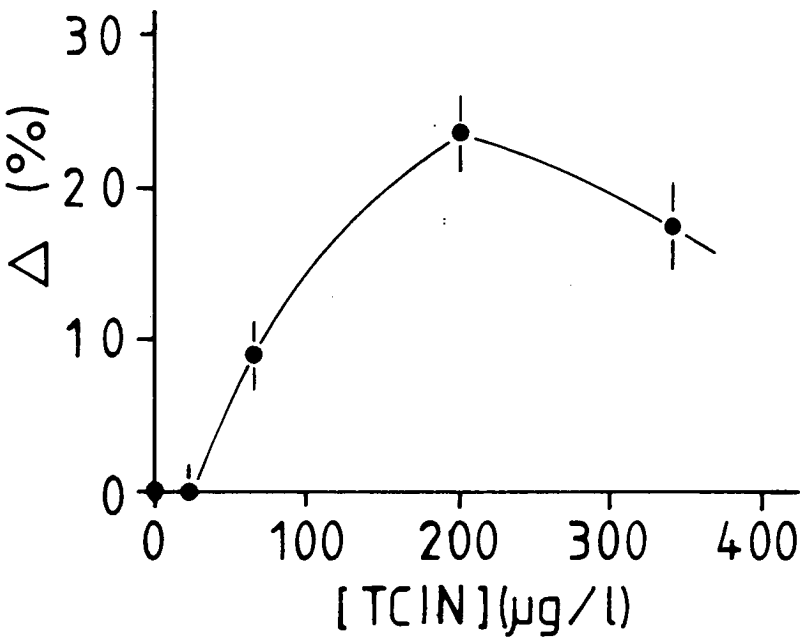
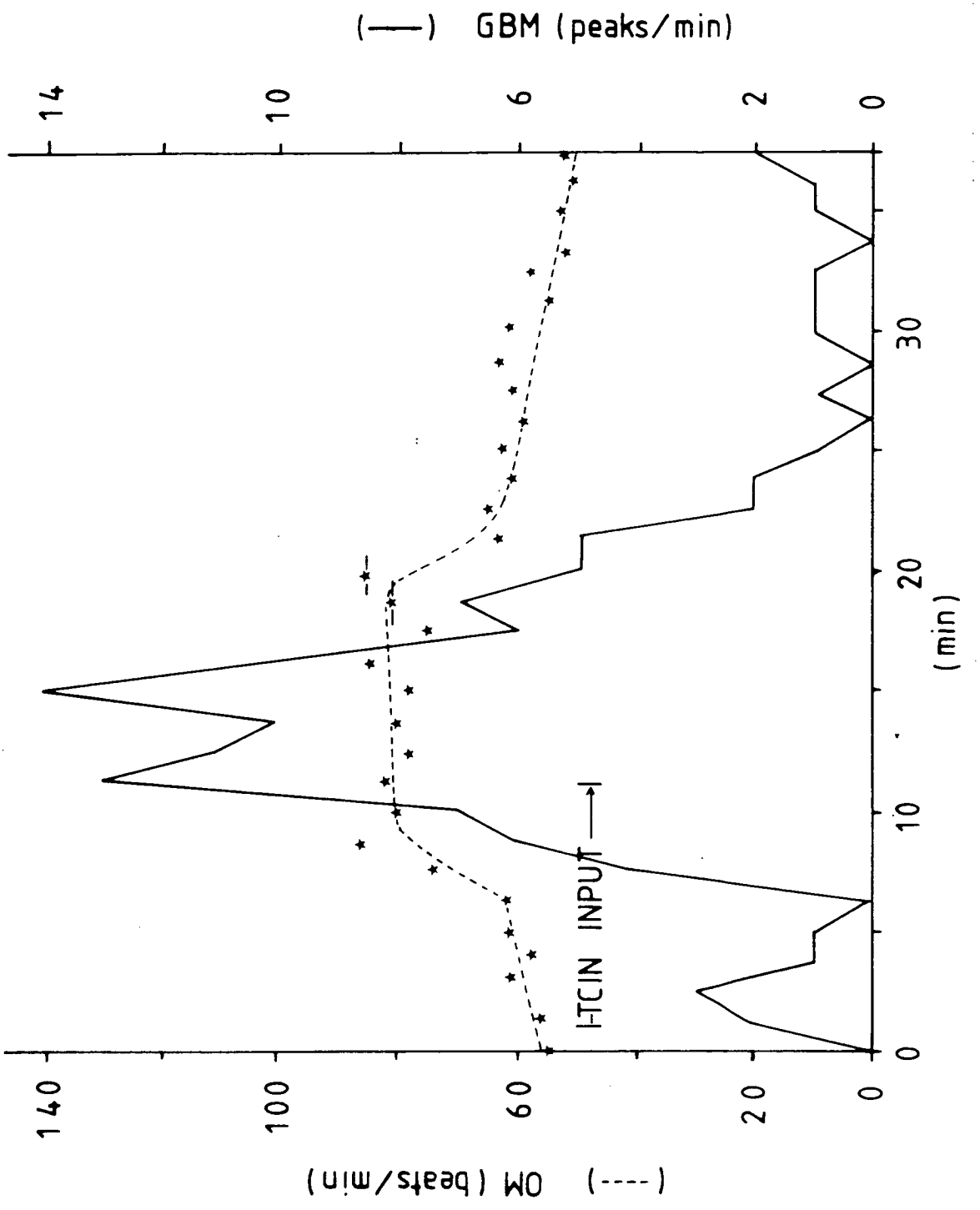


Fig. 3.6 Ventilation rate response of S. gairdneri (N = 5) to a 9 min exposure to TCIN. Response time = 30 min. Δ = % difference from controls. (I) = S.E.

Fig. 3.7 Typical response profile of S. gairdneri to a short pulse of TCIN at 200 ug/l (15° C).
OM = opercular movements.
GBM = gross body movements.
-*- = bursts of rapid ventilation.
See text for details.


(——) GBM (peaks/min)
14
10
6
2
0
30
20
10
0
(min)
I-TCIN INPUT
140
100
60
20
0
(----) OM (beats/min)

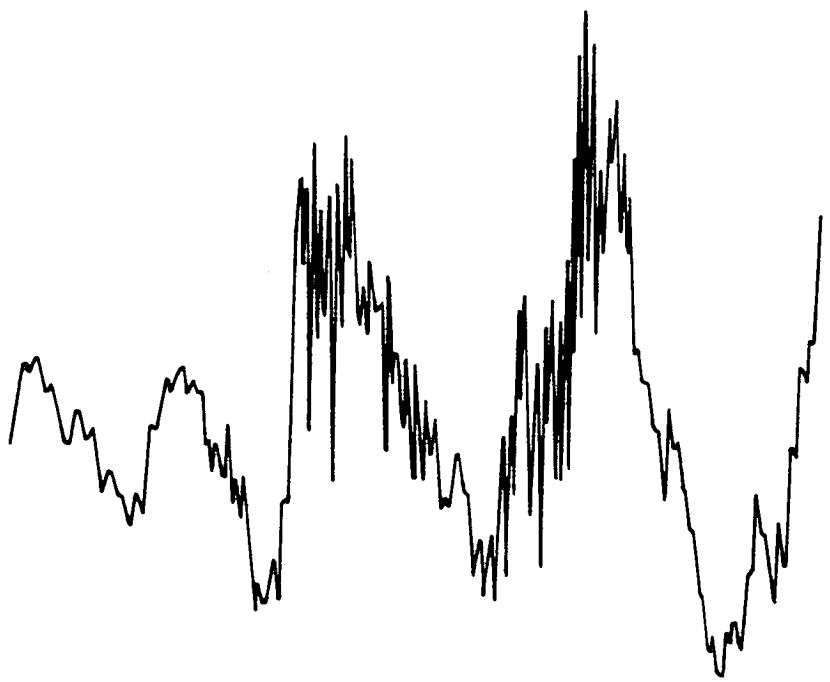


Fig. 3.8 A typical pulse of rapid ventilation of S. gairdneri exposed to 300 ug/l TCIN.

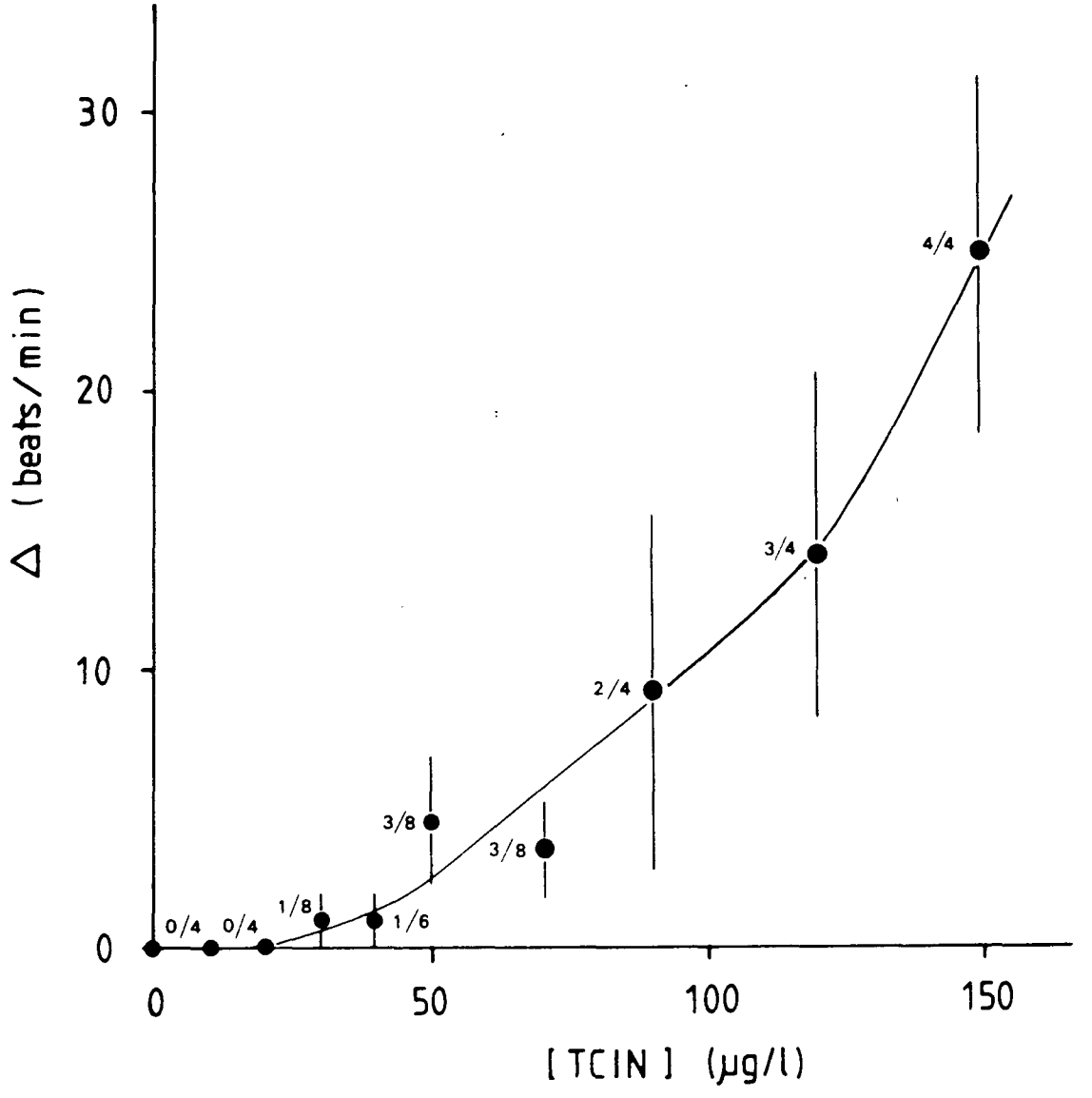


Fig. 3.9 Ventilation rate response of S. gairdneri to a 2 h exposure to TCIN. Δ = mean difference in rate (beats/min) from control period. Proportion of fish responding ($p < 0.05$) at each concentration is shown. (I) = S.E.

could decrease the TCIN levels at which such responses are shown at low oxygen concentrations. This was not tested in this work.

3.3 DESIGN OF A SYSTEM FOR COLLECTION, STORAGE AND PRIMARY ANALYSIS OF FREQUENCY DATA

3.3.1 INTRODUCTION

Data from the electrode chambers commonly used in fish ventilation frequency response experiments is generally in the form of a complex waveform consisting of a number of components (Fig. 3.10). The unfiltered signal is composed of low-frequency (0 - 0.5 Hz) gross body movement signals, including caudal fin movement, median frequency fin movements (0.5 - 1 Hz), and opercular movements (1 - 3 Hz). Unfortunately, these frequency ranges can frequently overlap. The overlap is much reduced if fish are quiescent and not involved in active movement.

The changes in impedance caused by fish movements between the cell electrodes are amplified prior to collection, generally on a chart recorder. The signal may be filtered to eliminate the lower frequency, high-amplitude signals. Usually opercular beats are hand-counted from the traced signal (Cairns and Garton, 1982; Van der Putte et al., 1983). Electronic devices cannot count the opercular beats on unfiltered signals due to large variations in the basal value of signal amplitude. Filtering eliminates this. However, a simple counting device depends on a threshold amplitude value in order to register a count. Both the natural variation in opercular movement signal strength and variation due to fish movement in the chamber preclude this approach as an absolute method of calculating the opercular frequency, since a proportion of the peaks are lost. The problems were noted by Gruber and Cairns (1981), who used a voltage-limited counting method. They found that signal loss was significant. However, this method could, within limits, be used in a comparative study.

It was decided that a digital-frequency analysis approach would be more effective and, with an on-line data manipulation computer system, would be more efficient, especially for long term studies. The

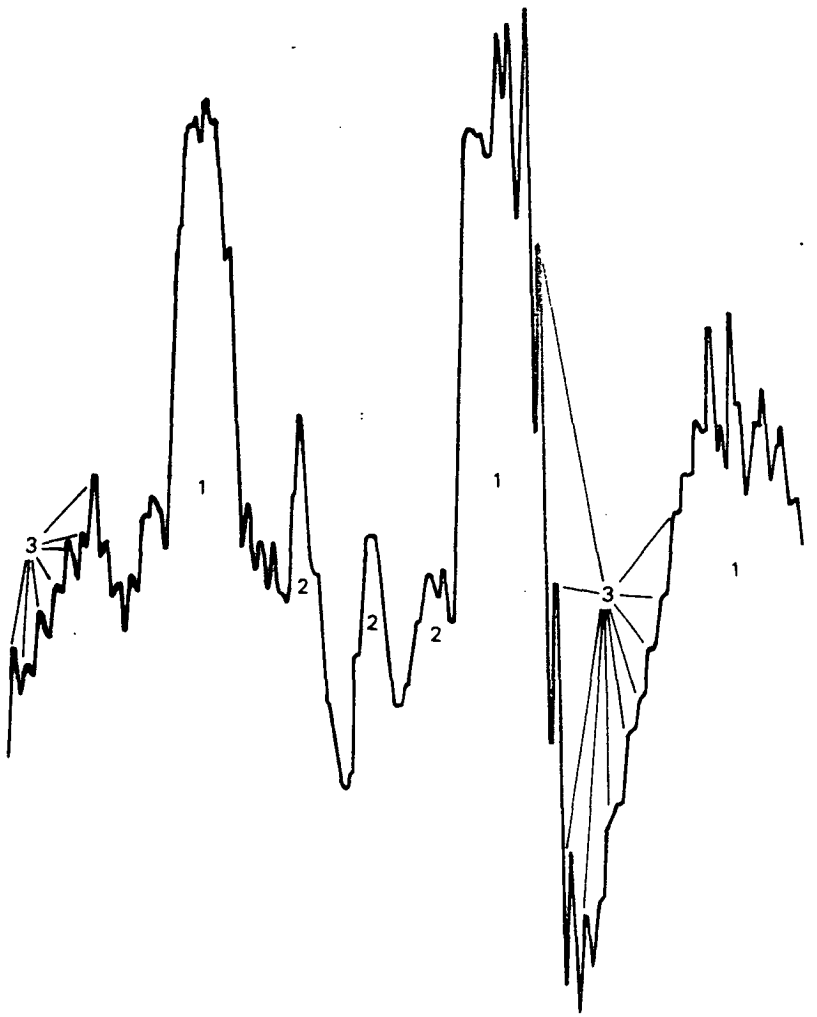


Fig. 3.10 Crude waveform of S. gairdneri movement signal from electrode cell system.

1 = Gross body movement signal peaks.
2 = Fin movement signal peaks.
3 = Superimposed regular opercular beat signal peaks.

approach was to convert the voltage or analog signal from the electrode chamber, after amplification and filtration, to a digital form which could then be analysed by Fast Fourier Transform (FFT) analysis, and converted to a power-frequency spectrum. This spectrum would give a high-power peak at the principal frequency within the complex waveform, corresponding to the ventilation rate frequency. The frequency values of spectral peaks from discrete time-intervals during an experiment could then be used directly in a computer-based analysis of experimental results, such as time-series analysis (Thompson et al., 1982).

FFT analysis involves the rapid conversion of a complex waveform into a series of component signals of different frequencies, and producing a power-frequency distribution. The waveform must be converted into a digital form amenable to the FFT calculation. It must also be divided into discrete time sets or units. Consequently, each FFT data set can represent an individual subsampled measure of ventilation frequency. The time span of these units depends on the frequency resolution required, the number of data points to be used by the FFT - generally a power of two such as 256, 1024 or 2048 - and the ability of the analog data collection device to collect data points at speed.

The theory of the FFT calculation is mathematically involved. It is sufficient for the purposes of this discussion to note that a complex waveform x(i), made up of a number (N) of single-frequency signals added over each other, can be represented by the integral:

$$x(i) = \sum_{k=0}^{N-1} y(k).e^{-j2\pi ik/N} \qquad i=0...N-1 \quad \text{Eqn. 3.1}$$

This forms a discrete pair with the integral:

$$y(k) = \frac{1}{N} \cdot \sum_{i=0}^{N-1} x(i).e^{j2\pi ik/N} \quad k=0...N-1 \quad \text{Eqn. 3.2}$$

which is called the Fourier Transform of y(k), where y(k) is the k^{th} sample of a time-series of a digital signal (converted from the analog waveform) consisting of N samples. The FFT calculation performs the transformation of the digital data waveform collected as the time-series

in equation 3.1, and converts it to a power-frequency spectrum represented by equation 3.2. A brief introduction to the FFT is afforded by Hughson (1978), and an outline of the mathematical details is given by Fagan (1979). A more detailed discussion of the FFT is given by Cooley et al. (1969).

The FFT is performed on a finite sample, or a distinct period of time, of the waveform signal, and this has obvious advantages in the running of a ventilation-frequency response experiment where each FFT data group collected can be used as a piece of time-series data for averaging or co-analysis with similar data from the toxicant-exposed or control periods.

3.3.2 MATERIALS AND METHODS

Data collection, analog - digital conversion and storage

A self-contained Analog to Digital (A-D) converter system designed by Trevitt (1981) was built which had the following general features:

1) Six optional input channels.
2) An analog-digital converter which collected 512 or 1024 analog samples at 0.5 - 20 samples/sec, converted them to digital values, stored them, and moved onto the next channel, cycling through them every 0.5, 1, 2 h or continuously.
3) A tape-storage facility enabling immediate storage of each sampling period on tape in digital form.
4) A read-out/play-back facility which enabled the data to be played out into an interfaced PDP - 11 computer for subsequent data manipulation.

The above system was used to collect analog waveform data from the flow-through electrode-cell system described previously during the low-level 2 h exposure experiment (3.2.2), in order to test the effectiveness of data collection and FFT data manipulation and compare it with the hand-counting technique for estimation of opercular rhythm frequencies. The outputs from the electrode-cells were amplified using

a pre-amplifier (10,000 x), filtered at 0.5 Hz (lower cut-off), and amplified again using a variable gain amplifier. Signal voltage span was optimised between $\pm$ 2.5 V, by observing on a chart recorder trace and manually setting the signal strength, prior to sample collecting.

Time of sampling of 1024 sample count sets for the A - D converter was 1.5 min. Coupled with 30 s recording time for data transfer to tape, this represented a 2 min collection interval. For a long-term experiment, collection of 1.5 min data every 30 min is suitable.

A set of programs were written in FORTRAN to process the data collected from the A - D converter. Data was transferred from tape to disc storage on a PDP - 11 computer using a transfer program : PETE (Appendix 4). The individual data sets of 1024 points could be plotted to reproduce the sampled waveform.

Data sets were individually processed by an FFT program package (Appendix 4). This output could be printed or plotted to give peaks of the power-frequency spectrum. The main power peak was taken to represent the principle ventilation frequency for the 1.5 min period sampled. No further data manipulation programs were written.

3.3.3 RESULTS

A typical plot of a digital waveform data set is shown in Fig. 3.11 a. A typical 1.5 min analog waveform from a chart trace can be compared with it (Fig. 3.11 b). Continuous data sampling theory (Nyquist theory) states that $N \geqslant 2BT$ data samples per signal of frequency B (c/s) and sampling time T (s) are required for effective signal resolution. The sampling rate of 11 samples/sec for 1 - 2 Hz signals represents 5 - 11 samples/cycle and is above the Nyquist requirement of $\geqslant$ 2 - 4 samples/cycle. For wavelengths $>$ 6 Hz the resolution of the sampling process drops rapidly to zero giving an upper frequency limit of resolution.

Typical FFT data sets plotted as power-frequency spectra are shown in Fig. 3.12. The highest power peaks are taken as the principal opercular rhythm frequencies for the sampling period. Visual observation and chart recordings gave very good agreement with the frequency peaks. The FFT procedure was found to improve estimation of

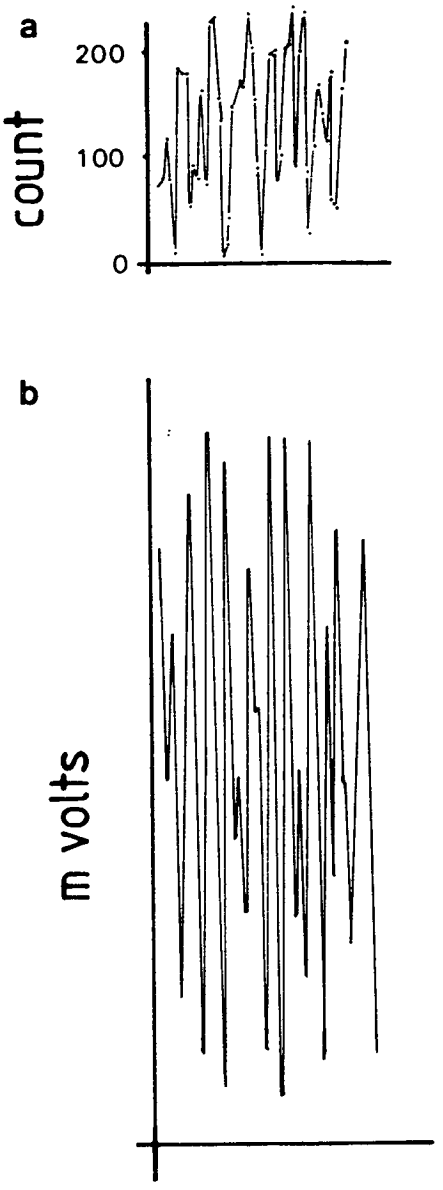


Fig. 3.11 Opercular rhythm waveforms of S. gairdneri.

a Trace of digital waveform data collected and converted by the A - D convertor.

b Similar waveform derived directly from chart trace of the output of the electrode cell system.

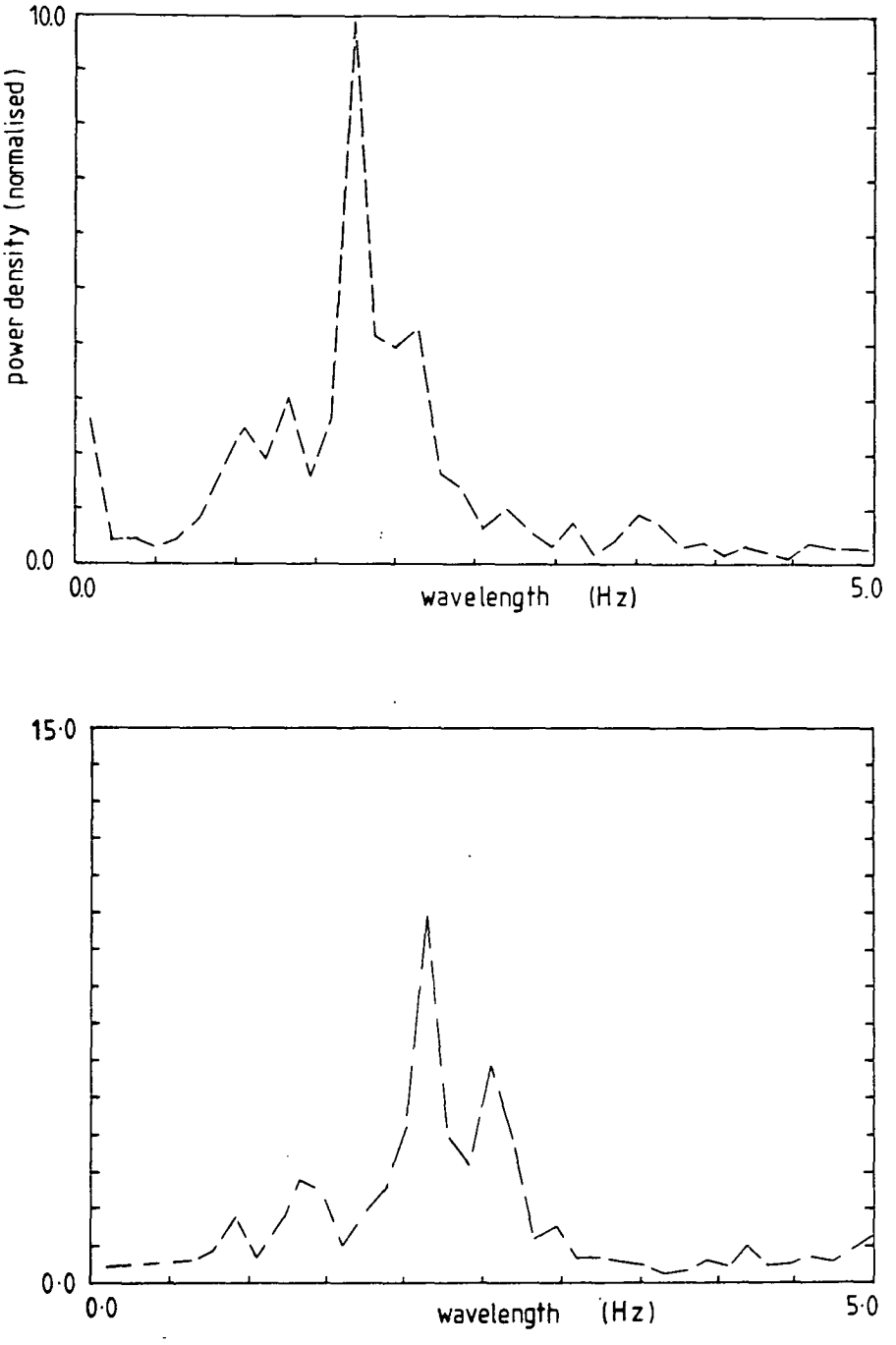


Fig. 3.12 Typical traces of power - frequency spectra of electrical signals of S. gairdneri after FFT of a single observation of the signal.
(File SCN 12. P17,a; File SCN 11. P15, b).

the frequency by "unscrambling" of over-lying waveforms of similar wavelengths, and filtering random peaks,all of which may be included in hand and electronically counted data sets.

High-power peak frequency data for 40, 50 and 70 ug/l exposure periods in the previously described low-level respiratory response experiment (3.2.2) were averaged for each fish (N=5) and are compared with data obtained by hand counting of the same signal in Table 3.5. The mean beat/min response difference agrees with the hand-counted value. The single significant response corresponds to that fish giving the highest response in the hand-counted data. It, therefore, appears that FFT and hand-counted data agree well, although a more extensive comparison is required.

3.3.4 DISCUSSION

Practical limitations occurred with regard to the ventilation frequency response experiments. The A-D converter requires signal voltage strength bounds, in this case of $\pm$ 2.5 V. If an unfiltered signal is sampled, the large amplitude variations caused by body movement signals must be included within the voltage range. The amplitude of the opercular movement signals are, however, much smaller (5-10%). In order to maximise the resolution of the FFT analysis for opercular rhythm frequencies, the number of sample points that can be used for transformation in the frequency ranges attributable to opercular rhythms must be maximised, as should the amplitude of the opercular rhythm signal. Consequently, the accuracy of the FFT analysis with regard to the opercular rhythm signals when analysing data of which 90-95% is attributable to body and fin movements is greatly reduced. Filtering then becomes mandatory in order to eliminate the large baseline signal variations and allow maximisation of opercular rhythm signal strength. A filter cut-off frequency of 0.5 Hz was selected. Data above this frequency was counted after increasing amplification to maximise the voltage signal scan.

The data filtering cut-off limits represent the major limitation of this counting method. As indicated above, under certain circumstances body, fin and opercular movement signals can overlap in frequency. This can occur when fish are acutely stressed and all

Table 3.5 Comparison of estimates of ventilatory frequency differences from control (Δ) in TCIN exposed S. gairdneri by hand-counting and FFT procedure

TCIN concentration (ug/l)	Δ, SE; Proportion responding (bpm)	
	FFT	Hand-counting
40	1.4, 0.7; 1/6	1.0, 1.0; 1/6
50	7.3, 3.6; 2/8	4.6, 2.3; 3/8
70	4.8, 2.9; 3/8	3.6, 1.8; 3/8

movements become rapid and erratic, or when opercular movements are slow. This problem also applies when data is hand-counted, where a degree of subjectivity must be used in order to count opercular signals. However, data of this type can be recognised and rejected with the A-D converter sampling method. If the opercular rhythm signal is slow and comparable to fin and body movements, a judicious choice of filter cut-off will cause the signal to drop below the cut-off limit and not be counted. If body and fin movements increase to the frequency of the opercular rhythm, their greater signal amplitude will override the voltage limits set by the A-D converter input. A data test can be incorporated to eliminate data sets in which a high proportion of counts are at the upper and lower voltage limits. This type of signal confusion generally occurs spasmodically and under acute stress conditions. Since most ventilation frequency response experiments are run with pollutant concentrations much lower than those expected to frequently cause such responses, a data elimination test should not remove too many data sets. Data filtering is commonly used in ventilation-frequency response experiments (Van der Putte et al., 1982).

A further problem with the tape-recording data storage system is related to the quality of the recording system itself. The FFT package is designed to reject data sets in which erratic datapoints occur, i.e. samples which are not sequentially consistent with the waveform. Such "samples" may be produced by tape imperfections or random electronic noise recorded during the sampling period. Loss of data sets by this process can be quite high on occasion. It may be effectively reduced by using good quality tape and recording heads, and by reducing ambient electric noise (light switching etc.). Good electric shielding of the electrode cells is imperative with regard to this phenomenon in order to maintain signal integrity.

Further data manipulation programs envisaged to fully evaluate a ventilation-frequency response experiment are as follows:

1) Data screening program.

Aim:

to collect peak power in the FFT spectrum, and to place these discrete values in a time-series array for each channel/fish.

2) Data analysis.
Aim:

a) To perform time-series analysis by the method of Thompson et al. (1982).

b) To output time and extent of significant opercular-rhythm differences in exposed fish from controls. Control data sets are previous sets from the same channel/fish.

Such a system could be operated on-line if the recording phase of the A-D converter collection system can be converted to a data-transfer phase into the dedicated computer at the conclusion of each sample period, and if the disc storage and data processing as detailed are performed on each incoming data set, and output continuously.

In conclusion, the above system appears to be an efficient means of sampling, retrieving and recording data during a ventilation-frequency response experiment. It avoids the extensive use of chart paper and avoids subjective and tedious hand-counting of data peaks. Loss of data by fluctuations in signal amplitude caused by fish movement within a respiratory monitoring cell is frequently a problem in electronic peak counting systems, where minimum voltage constraints are set on the signals (Gruber and Cairns, 1981). Such losses are eliminated when using the FFT approach which analyses data on the basis of frequency components.

This system allows data sets to be sampled, stored and analysed by computer with a minimum of effort.

CHAPTER 4

TOXICOLOGICAL EFFECTS AND BIOCONCENTRATION OF CHLOROTHALONIL IN SALMO GAIRDNERI

4.1 TOXICOLOGICAL EFFECTS

4.1.1 INTRODUCTION

Any study of the toxicology of a toxin in an organism should be accompanied by detailed descriptions of the various symptoms of toxicosis, either behavioural, histological or biochemical. These descriptions aid in the diagnosis of the source of the toxicosis, and permit the making and testing of hypotheses about the nature of toxic action and the modes of detoxication and adaptions to exposure available to the organism. This section describes the symptoms of behavioural toxicosis for Salmo gairdneri, Galaxias maculatus, G. truttaceus and G. auratus on exposure to TCIN. The effect of TCIN exposure on gill anatomy, blood cell volume, tissue lactate growth and bile colour in S. gairdneri are also described.

Gill damage caused by heavy metal pollutants is well documented, especially for zinc (Alabaster and Lloyd, 1982). Zinc causes mucal coagulation and gill damage which results in the inhibition of gas diffusion across the respiratory surface. This is considered one of the prime causes of the toxic action of that metal in fish. General histological examinations have indicated that many xenobiotics can cause gill damage. Conditions such as hyperplasia (gill lamellar fusion), hypertrophy, necrosis and membrane rupture are frequently encountered. These are generally reported as being primarily due to organic or physical causes such as alkalinity (Daye and Garside, 1976), ammonia (Burrows, 1964; Reichenbach - Klinke, 1967; Flis, 1968; Smart, 1976), cadmium (Bilinski and Jonas, 1973), copper (Pequignot et al., 1975), zinc (Alabaster and Lloyd, 1982), anionic detergents (Abel and Skidmore, 1975) and suspended solids (Alabaster, 1972), than from the action of organic xenobiotics (Johnson, 1968; Hattula et al., 1978).

Johnson (1968) stated that, at the time of his review, "histological effects of pesticides remain largely undefined and most of the recent work is inconclusive". The situation has improved only marginally during the last fifteen years. Few detailed histological studies on pesticide-induced damage have been carried out and, those which have, generally concentrate on liver damage (Racicot et al., 1975; Owen and Rosso, 1981). No quantitative accounts of gill damage by pesticides have been published. Due to the possibility of a respiratory involvement in the toxicology of TCIN in fish, it was decided to investigate the possibility of TCIN induced gill damage.

Hughes and Perry (1976) detailed a method for the quantitative assessment of gill structural changes in S. gairdneri. Their morphometric technique would be applicable to any gill with the structure and uniformity occurring in salmonids. A variety of parameters can be investigated by the assessment of volume and surface area ratios within the secondary lamellar structure. An estimate of the diffusive capacity of the gills, Dlam, can be made and compared between gills exposed to various conditions. Gill damage on exposure to low levels of TCIN over 24 days was assessed by this method in S. gairdneri.

Haematocrit (Hct) is the percentage packed red-cell volume in the blood. Its value is dependent in the nutritional and metabolic state of the animal. Snieszko (1960) reviewed the uses of Hct values for detection of "abnormal" and pathological states in fish, also reporting that many North American hatcheries used microhaemotocrit as a part of a year-round quality control program. In cases of environmental pollution Hct has often been found to rise due to the stress induced by exposure to toxins (Sniezsko 1960; Johnson, 1968; Reinitz and Rix, 1977; Darrow et al., 1978, Swift, 1982), where changes in salmonid Hct under anaerobic conditions have been shown to be caused by erythrocytic swelling (Soivio et al., 1974)

The normal values of Hct for salmonids generally fall within a relatively narrow range (Darrow et al., 1978), although they vary from stock to stock (Cameron, 1971). A review of blood parameters used for the health assessment of freshwater fish was published by Blaxhall (1972). He gave typical values of S. gairdneri haematocrit as being within the range of 45-53%. Haematocrit values can vary due to changes in blood plasma volume, erythrocyte number and erythrocyte volume.

Since an interaction between TCIN toxicity and oxygen levels was observed (Chapter 3), and since a response of the respiratory system was suspected, the relationship between TCIN exposure and blood cell volume (Hct) was investigated. The responses of Hct values to exposure to compounds other than TCIN, which were structurally related, lindane and pentachlorophenol (PCP), two well known pesticides, and DAC3701, were also investigated for comparative purposes.

Induction of high lactate levels in tissues of animals undergoing hypoxia is a well known phenomenon. Black et al. (1962) demonstrated that muscular activity in S. gairdneri caused a rapid increase in lactate and pyruvate levels in muscle tissue which only returned to resting levels 8 h after cessation of activity. They reported the mean resting level in S. gairdneri to be 187 $\pm$ 12.5 mg/100g muscle lactate (N=19) for 260 g fish. This increased to 380 mg/100g after 2 min strenuous exercise and attained a plateau of around 420 mg/100g after 5-15 min. Blood levels were much lower and showed a different response, increasing steadily with time of exercise. Similar responses were obtained for S. gairdneri muscle lactate levels under acute anoxic and prolonged hypoxic conditions by Smith and Heath (1980).

In order to test the hypothesis that interference with respiratory functioning by TCIN causes local tissue hypoxia leading to death, it was decided to analyse TCIN exposed fish for muscle lactate levels and compare them to controls. Darrow et al. (1978) followed a similar line of enquiry to investigate the possible respiratory mode of toxic action of n-decanohydroxamic acid to Salvelinus fontinalis. They analysed a number of blood parameters including Hct and serum lactate levels. In a 5 h exposure to the toxin the fish showed an increase in Hct, which was related to an increase in cell volume. A thirteen fold increase in serum lactate occurred indicating that anaerobic glycolysis had occurred.

Growth has occasionally been used as an indicator of environmental stress (Webb and Leduc, 1981). During acute toxicity trials it was observed that fish exposed to TCIN showed a marked loss of appetite. It was decided to investigate the effect of long-term low-level exposure to fed S. gairdneri on growth parameters.

In a preliminary examination, S. gairdneri exposed to TCIN displayed a distinct colour change in the bile. Changes in biliary colour have been reported by Talbot and Higgins (1982) in fish which have been starved for periods in excess of 24 h. They proposed that biliary colour in salmonids could be used as an indicator of the time since the last feeding event.

In the salmonids, the gall bladder is a transparent sac lying within a pocket formed by the left lobe of the liver, the stomach and the hind-gut. Unless a feeding stimulus occurs, it stays full for periods of up to 12 days, but it is evacuated almost completely on feeding. Changes in colour since last feeding event may be due to build up of waste metabolites from the liver or a change in the biliary pigment constituents. It was decided to investigate TCIN induced bile colour changes in more detail.

4.1.2 MATERIALS AND METHODS

Unless otherwise indicated, Salmo gairdneri (8–11 g) were supplied, acclimatised and fed as detailed previously (3.1.2).

Toxicosis symptoms

Detailed observations on the behaviour and appearance of fish exposed to TCIN at varying concentrations up to 50 ug/l were recorded during the LC50 experiments described in Chapter 3, for Salmo gairdneri, Galaxias maculatus, G. truttaceus and G. auratus.

Gill morphometric analysis

Six groups of five fish each were exposed to the following TCIN concentrations: 0, 0, 1.15 and 2.0 ug/l, in a daily-replacement static exposure test in floating plastic bags containing 52 l aerated water at 10–13° C. After exposure all fish were slaughtered, and gills fixed in buffered formalin. The central section of the central gill arch was selected from the left and right side of each fish. The tissues were dehydrated in an alcohol series and embedded in Spurr's medium sections of 1 um thickness, were cut and stained with 1% toluidine blue in 4% borax solution, and examined at 1000 x magnification. The

projection microscope facility recommended by Hughes and Perry (1976) was not available, and counts were made under an ordinary high-power microscope. The Merz grid, imaged on microfiche, was placed on the underside of a graticuled eyepiece which was used to measure blood-water barrier distances. Counts of line-membrane intersections and point-tissue locations were made at five different locations on each section. One representative section was counted per gill after being selected for correct orientation and lack of artefacts. This led to 50 replicate grid counts per fish treatment group. Mean harmonic blood-barrier distances, τ , were calculated in um on the basis of 10 distances measured per section, giving 100 replicate measurements per fish treatment group. Both of the exposure groups were processed, counted and analysed, with a separate control group, to minimise preparational artefacts.

Counts and ratios calculated from each section were as shown in Figs. 4.1. and 4.2 on a diagrammatic section with Merz grid superimposed.

Haematocrit

Groups of four S. gairdneri were exposed to concentrations of TCIN of 0, 10, 20, 30, 45 and 50 ug/l for varying periods of time (intervals of 6 and 12 h) at 13-16° C. Fish were pithed and blood samples taken directly into microhaematocrit tubes after severing the tail. Anaesthetic was not used as it has been shown that MS222 affects Hct values (Reinitz and Rix, 1977). After centrifugation for 15 min at 5000 rpm, the percentage volume contribution of red cells was calculated.

Groups of four S. gairdneri were also exposed to 16 ug/l lindane, and 157 and 95 ug/l PCP for 3 days in a static daily-exchange bioassay. An acetone-only control group was included. Acetone carrier concentrations were $\leqslant$ 20 mg/l. Hct levels were measured as above.

Groups of five S. gairdneri from the DAC3701 LC50 experiment (Chapter 3) were analysed for Hct at the termination of exposure to 0, 20, 45, 60, 104 and 200 ug/l for 144 h.

Lactate levels

Two groups of S. gairdneri were exposed to 0 and 50 ug/l TCIN,

Fig. 4.1 Merz grid. Overlay on Fig. 4.2.

Fig. 4.2 Typical gill lamellar section of S. gairdneri. Parameters defined for morphometric analysis:

Areas within the section

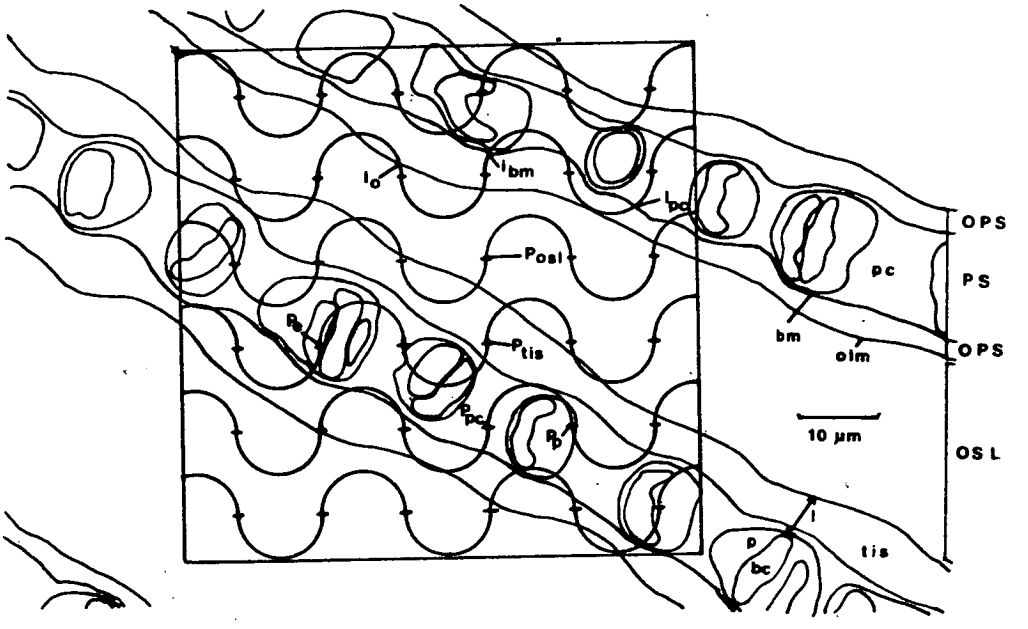
OPS = outside pillar cell system.
PS = pillar cell system.
OSL = outside secondary lamella.
pc = pillar cell.
bm = basement membrane.
olm = outer lamellar membrane.
tis = outer tissue area.
p = plasma.
bc = blood cell.
l = blood barrier thickness.

Grid counts

Ibm = intersection of line with basement membrane.
Ipc = intersection with pillar cell.
Io = intersection with outer membrane.
Posl = point between lamellae.
Ptis = point within tissue area.
Pe = point within erythrocyte.
Pp = point within plasma.
Ppc = point within pillar cell.

Resulting ratios

$Ve/VSL = Pe/\Sigma p - Posl$
$VOPS/VSL = Ptis/\Sigma p - Posl$
$VSL/VLR = \Sigma p - Posl/\Sigma p$
$SI/SO = Ibm + Ipc/Io$
$I_I = Ibm + Ipc$
$Ao = A.Io/I_I$
$Hct = Pe/Pe + Pp$
$\mathcal{T}$ = harmonic mean of l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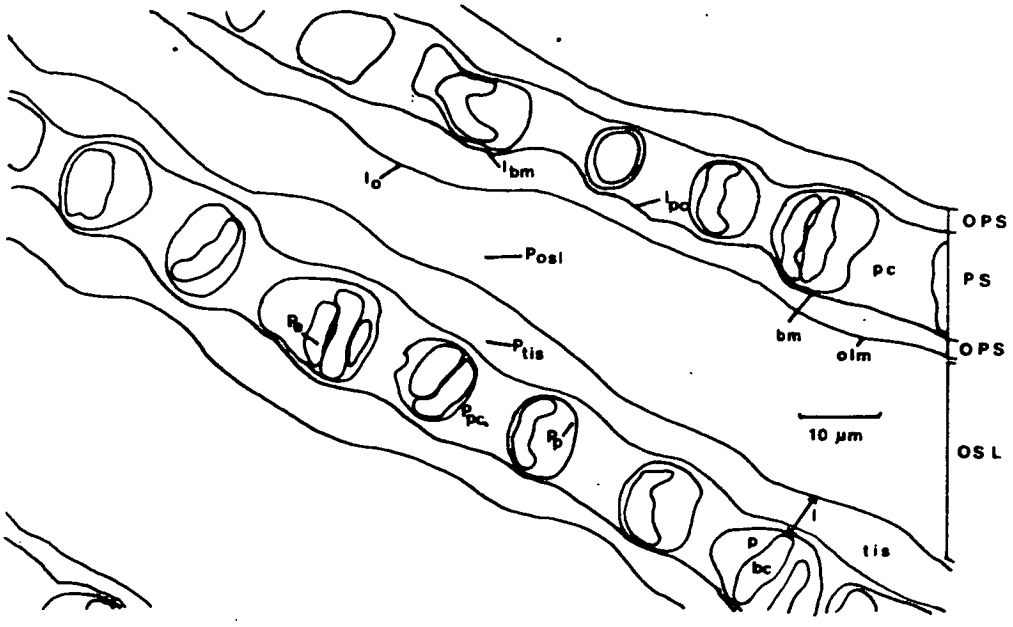
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and samples of 4 fish were sacrificed at 0, 2, 18, 26 and 48 h . Due to the rapid increases in lactate levels noted by Black et al. (1962) within 2 min of exercise, fish were taken with a hand net, and placed as rapidly as possible ($\leqslant$ 15 s) into liquid nitrogen. Fish were stored in liquid nitrogen until required. They were then dissected frozen and the last third (1-1.5 g) of dorsal muscle tissue was hand-ground at 0-2^{o} C in a pestle and mortar for 10 min in 10 ml 0.6 M TCAA in 0.3 M HCl. Lactate levels were analysed in 0.1 ml aliquots of homogenate supernatant using a lactate assay kit from Boehringer (GmbH) after centrifugation at 2000 g per 10 min.

Growth

Fish were exposed to 1.15 and 2.0 ug/l TCIN for 21 days with feeding. They were weighed and their length measured, along with controls, before and after the exposure period.

Biliary colour change

S. gairdneri from the previously described high-oxygen level LC50 exposure experiment (Chapter 3) were dissected and their gall bladder colour and fullness recorded. Visible wavelength spectra were determined on samples diluted with distilled water (5:1), for typical bile colour.

4.1.3 RESULTS

Toxicosis symptoms

All fish showed marked lethargy on exposure to TCIN. The degree of lethargy increased with time and concentration of exposure. Salmo gairdneri showed normal startle reactions at exposure levels less than 8.7 ug/l, whereas for G. maculatus, G truttaceus and G. auratus, startle reactions were normal below 8.8, 9.0 and 13.3 ug/l exposure over 96 h .

In S. gairdneri, loss of startle reaction was followed by reduction of activity, and station holding at the water surface or tank bottom. A gradual bronzing of skin colour occurred, especially in facial patches and around the lateral-line. Reddening of fin bases was also observed. Permanent lethargy was followed by loss of righting

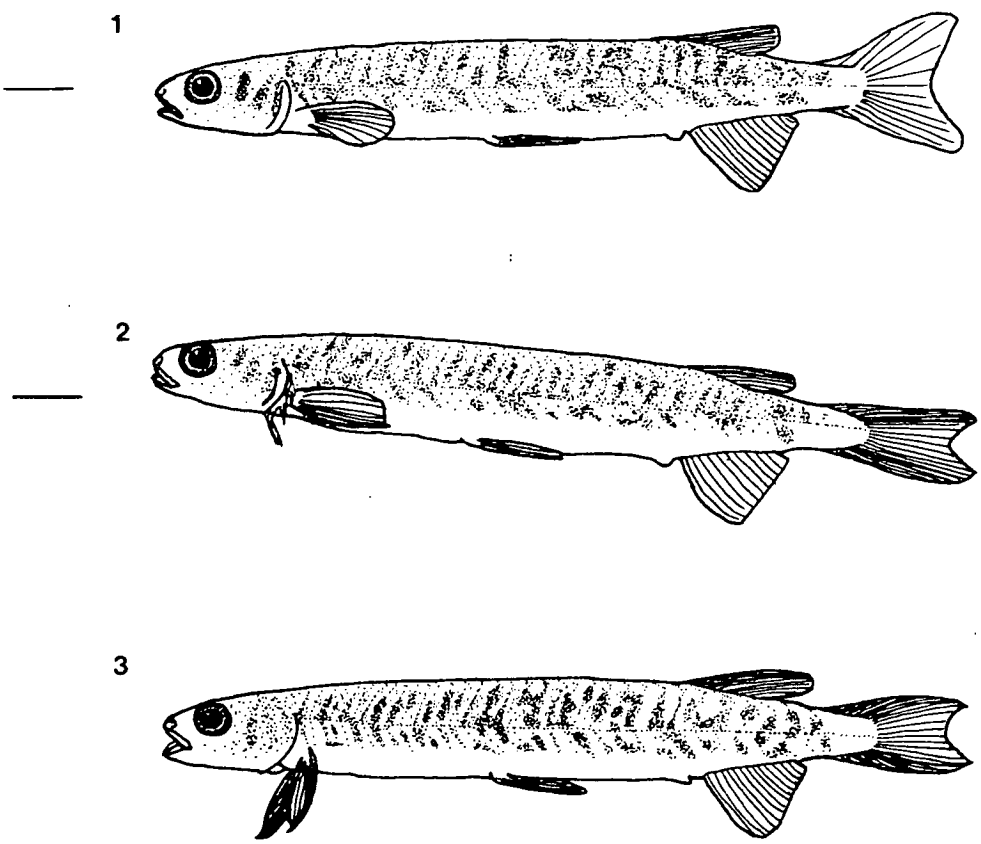


Fig. 4.3 TCIN toxicosis in Galaxias maculatus.

1: First stage of fin collapse.

2: Second stage of fin collapse with mucal clotting.

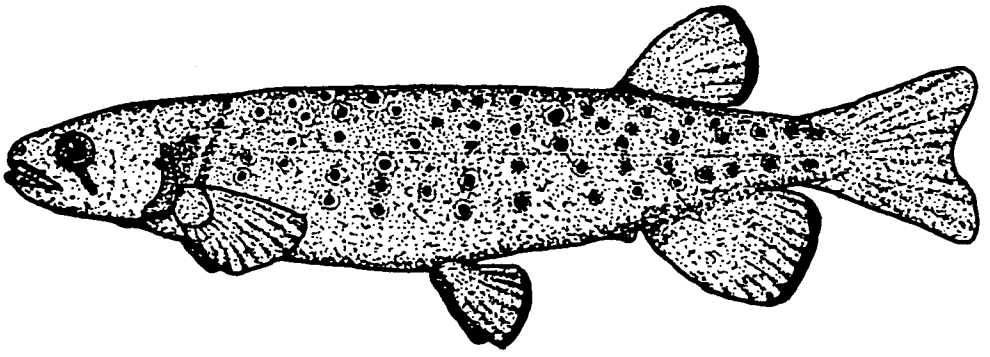
3: Fish at death.

Fig. 4.4 TCIN toxicosis in Galaxias truttaceus.

1, 2, 3 = First, second and third stages of fin collapse.

4 = Fourth stage, with loss of station holding ability.

1



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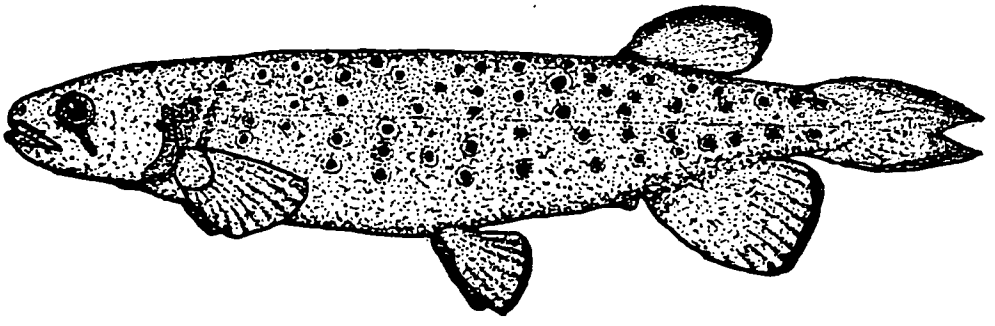

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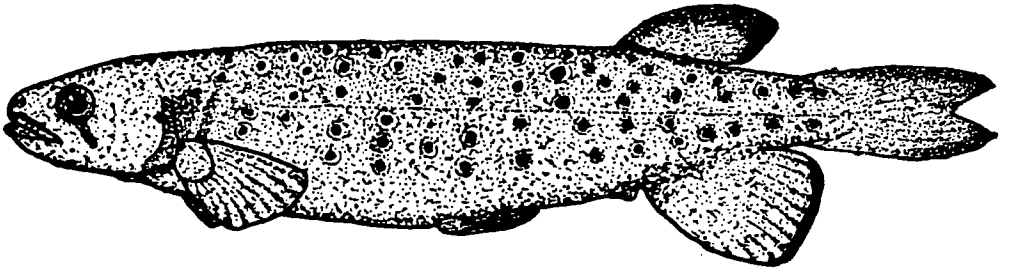

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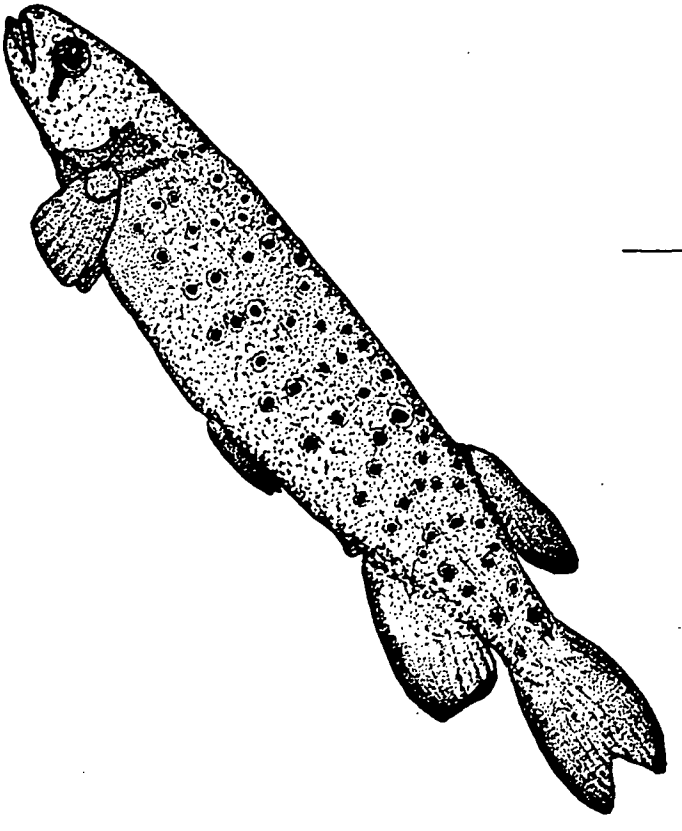


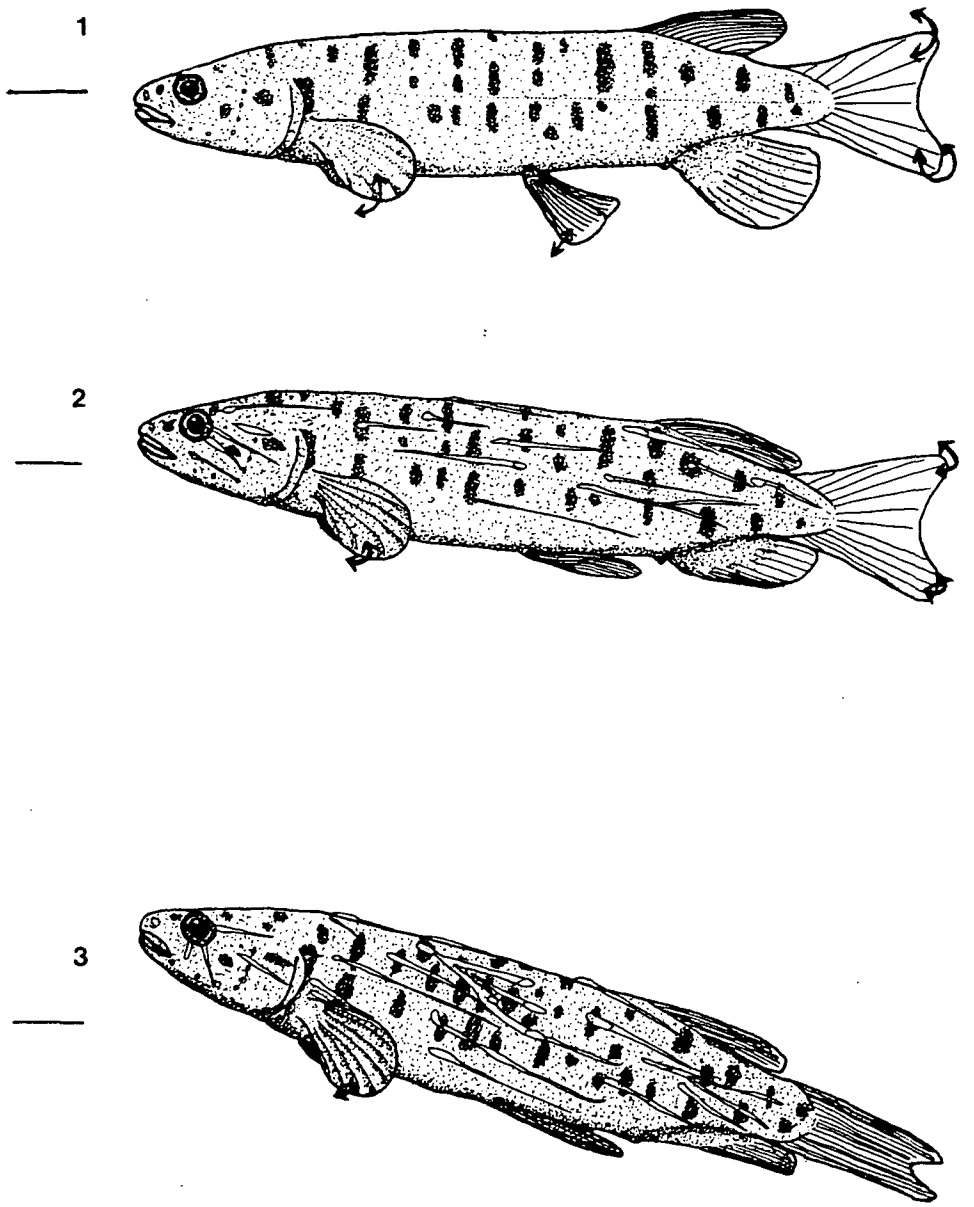


Fig. 4.5 TCIN toxicosis in Galaxias auratus.
1,2,3 = First, second, third stages of fin collapse, loss of motion and extent of epidermal mucal clotting.

ability and death. Dead fish were a pronounced bronze colour. No fin collapse occurred.

In all galaxiid species, the onset of lethargy was accompanied by varying degrees of fin collapse, often in the order : pelvic/ dorsal/ anal/ caudal. Pectoral fins never fully collapsed, though they were restricted in mobility. Pronounced skin mucal clotting occurred prior to death, though no colour change was observed. Various stages of toxicosis are shown in Figs. 4.3 - 4.5 for the galaxiid species.

Gill morphometric analysis

The values of calculated surface area and volume ratios, and Dlam are shown in Table 4.1. As $^{So}/_{SI} = 0.970 \pm 0.226$ and 1.099 ± 0.276 for the control groups tested, ie $^{So}/_{SI} = 1.0$, then it can be used as a substitute for the ratio $^{Io\ exp}/_{Io\ con}$ in the calculation of Dlam (Hughes and Perry, 1976). The two control groups were not significantly different in any values, and their results have been combined. They agree well with control values reported by Hughes and Perry (1976).

Only fish exposed to 2.0 ug/l showed significant differences from controls, although some of the trends (increase in τ, decrease in $^{Ve}/_{VSL}$ and $^{VOPS}/_{VSL}$) can be observed in fish exposed to 1.15 ug/l.

Exposure to 2.0 ug/l caused extensive changes in gill parameters. Diffusive capacity, Dlam was reduced to 43% of that in controls ($p < 0.001$) This was primarily caused by an 82% ($p < 0.01$) increase in τ , the thickness of the blood-water barrier. No fusion of secondary lamellae was observed. There was a 16% increase ($p < 0.001$) in the volume of secondary lamellae within the lamellar region (the ratio $^{VSL}/_{VLR}$). This may have been related to increases in volume of components within the inner membrane. An 8% increase in tissue volume outside the pillar cell system occurred ($p < 0.05$). The relative erythrocyte to secondary lamellar volume ratio decreased to 48.9% of controls ($p < 0.001$). This difference can consist of a change in haematocrit and in tissue volume.

Haematocrit (Hct) can be estimated by comparing point counts of blood cells and plasma space. This estimate can only be made with large replicate numbers since it was found that a low number of points per grid fell within the blood system (12%). Estimates of Hct are shown in Table 4.1. 2.0 ug/l treated fish had Hct values 65% of

Table 4.1 Gill morphometric parameters of S. gairdneri on exposure to low levels of TCIN for 24 days
* = values significantly different from controls

Morphometric parameters	TCIN concentration (ug/l)			
	0.0	1.2	0.0	2.0
Dlam - $cm^2/min/mmHg/kg$	0.1154-0.1338	0.1205-0.1446	0.1297-0.1156	0.0563-0.0675*
τ - um	4.291	4.403	4.130	7.532 *
Ao - cm^2/g	2.39	2.65	2.67	2.12 *
Ve/VSL	0.0543	0.0454	0.0968	0.0473*
VOPS/VSL	0.717	0.729	0.670	0.721
VSL/VLR	0.514	0.506	0.577	0.669 *
SI/SO	1.088	0.980	0.970	1.227 *
Pe+Pp/Σp	0.0929	0.0840	0.1062	0.1244*
Hct	47.64	50.98	48.51	31.55 *

control values, indicating acute anemia. This decrease in Hct and a 16% increase in lamellar value accounts for the 49% decrease in the ratio $^{Ve}/_{VSL}$.

There was a 27% ($p < 0.001$) increase in the ratio ($^{SI}/_{So}$) of inner to outer lamellar membrane surface area. Since total lamellar volume increased by 16%, it is likely that SI increased by 27% in fish exposed to 2.0 ug/l. This may have been related to increases in the volume of components within the inner lamellar membrane. Pillar cell volume was only increased by 2% ($^{VPS}/_{VLR}$), whereas total blood space increased by 17.1% (Pe + Pp/ Σp).

Haematocrit

Values of Hct at different exposure concentrations and times of exposure to TCIN are shown in Table 4.2. Hct was found to drop on exposure to TCIN. The magnitude of the decrease was proportional to time and concentration of exposure. When exposure concentrations were greater than 20 ug/l, Hct dropped significantly within 24 h .

Plasma of all controls showed no reddening due to haemolysis after centrifugation. Plasma of exposed fish showed distinct haemolysis whose intensity qualitatively followed a dose-response relationship.

Hct values of fish exposed to PCP and lindane are shown in Table 4.3, with the percentage 96 h LC50 values the exposure concentrations represent. Exposures to both pesticides caused significant increases in Hct values. Hct values of fish exposed to DAC3701 concentrations ranging up to 200 ug/l are shown in Table 4.3. There is a decreasing trend, although the differences were not significant.

Lactate Levels

Muscle lactate levels of exposed fish and controls are given in Table 4.4. There is no significant difference between the groups, even after exposure for 48 h.

Growth

Weights of fish exposed to 2.0 and 1.15 ug/l TCIN for 21 days showed no significant differences from controls (Table 4.5). Some indication of an increase in condition factor, K, was found where K is given by:

Table 4.2 Haematocrit depression by TCIN exposure

TCIN concentration (ug/l)	Time (h)	Hct (%)	p (from control)
50.0	12	40.4	<0.01
44.8	12,24	45.0,38.3	<0.01
30.8	12,24	50.2,42.6	n.s.,<0.01
19.9	24,72	53.6,46.2	n.s.,<0.01
10.0	24,72	57.2,46.8	n.s.,<0.1
0.0	0,12	53.8,54.5	---
	24,72	53.2,52.0	---

Table 4.3 Haematocrit values for S. gairdneri exposed to lindane and pentachlorophenol (PCP) for three days and to DAC3701 for six days, (N = 5)

Compound	Concentration (ug/l)	%LC50	Hct (SD)
lindane	16	10	67.25 (2.36)
PCP	95	63	54.00 (2.31)
	157	105	60.00 (1.00)
control	-	-	52.25 (2.75)
DAC3701	0		42.8 (3.7)
	45		40.6 (3.0)
	60		38.3 (8.7)
	104		35.7 (4.3)
	200		40.8 (4.7)

Table 4.4 Muscle lactate levels in S. gairdneri exposed to 50ug/l TCIN

Time (h)	Lactate (mg/100 g) (SE)
0	100.05 (35.3)
2	97.55 (27.6)
6	77.20 (23.2)
18	31.83 (11.46)
26	48.08 (14.55)
48	17.13 (1.14)

Table 4.5 Condition factor, K, of S. gairdneri exposed over 24 days to TCIN with feeding

TCIN concentration (ug/l)	N	K (SD)
2.0	5	0.0153 (0.0005)
1.2	5	0.0140 (0.0025)
0.0	5	0.0137 (0.0036)

Table 4.6 Biliary colour of S. gairdneri exposed to TCIN for four days

TCIN concentration (ug/l)	Biliary colour (N)
0.0	pale yellow (4)
	light green (4)
4.0	pale yellow (3)
	light green (5)
6.6	pale yellow (3)
	light green (3)
	dark green (1)
7.5	pale yellow (1)
	light green (6)
	dark green (1)
13.4	light green (2)
	dark green (4)
16.2	dark green (4)
	blue green (2)

$$K = W/L^3 \qquad W = \text{weight (g)}, \quad L = \text{length (cm)}$$

This was not significant at the 0.05 level (Table 4.5).

Biliary colour change

Colour changes in bile of fish exposed to TCIN are shown in Table 4.6. No quantitative measure of colour could be used, as visible wavelength spectra were featureless and showed absorbance generally decreasing over the range 370–550 nm for all samples, and no single wavelength could be used for comparative purposes. Indications of a slight peak or shoulder were obtained in most samples at 400–407 nm. The spectra did not allow colour changes to be related to particular constituent changes in the bile without more detailed examination. Bile of fish exposed to TCIN rapidly darkened and changed from a pale straw colour to a deep green. The intensity of the colour and the rate of change appeared to be dose-dependent.

4.1.4 DISCUSSION

TCIN exposure on a long-term basis at low levels, is shown to cause significant gill damage, reducing the diffusive capacity (Dlam) of the gills primarily by increasing the thickness of the blood-water barrier. Several other changes are coincident with the drop in Dlam. These include a significant decrease in blood haematocrit, based on volume ratios, and increases in both the secondary lamellar tissue volume and the lamellar blood space.

Increases in lamellar tissue volume and decreases in Hct are probably caused by the direct action of TCIN. An increase in lamellar blood space may be a physiological response to lowered diffusive capacity induced by these changes. Increases in gill blood flow have been shown to occur under hypoxic conditons (Soivio and Hughes, 1978) and this phenomenon is known to be under the control of andrenergic receptors (Payan and Girard, 1977). Measures of gill blood space by the morphometric method, one of the two other published uses of this method, in S. gairdneri were made by Soivio and Hughes (1978) when

comparing fish which had experienced normoxia and hypoxia. Consistent differences were not obtained predominantly due to small sample sizes. Only 19 grid counts in total were made to compare groups of two to four fish. Some differences were also observed when different fixatives were used. Soivio and Hughes (1978) cautioned on the use of uniform sample location within the gill arch and the use of fixatives. However, their high degree of variation cannot be divorced from small sample sizes. A detailed examination of the effect of heavy metals on the gills of S. gairdneri using this method was published by Hughes et al. (1979).

Values obtained here for Dlam and for control fish agree with those obtained for control S.gairdneri by Hughes and Perry (1976). Fish exposed to 2.0 ug/l TCIN for 21 days in this work showed the same type and close to the same magnitude of changes in these parameters as were obtained by Hughes and Perry for fish exposed to 2.0 mg/l nickel for an undisclosed period. $A.^{Io}/_{I_1}$ values are not as depressed by TCIN exposure as by exposure to nickel, indicating a greater decrease in outer lamellar surface area on nickel exposure. This is concordant with observations of lamellar fusion in nickel-exposed fish, but not in TCIN exposed fish.

Most stress-induced effects on Hct values involve transitory increases until physiological acclimation is achieved. The results obtained here indicate that TCIN causes acute and chronic anemia. The results obtained by measurement of blood haematocrit by centrifugation and by morphometrics are complementary, and the depression appears to be dose-related. The Hct depression is related to an increase in haemolysis. This haemolysis may be induced by TCIN in the blood stream caused by red blood cell rupture leading to depression in Hct. If the haemolysis is caused in vivo it may explain the reddening at fin-bases observed in toxicosis. It does not explain the bronzing colour, however, as this colour change is restricted to the skin and the mucus was not observed to be reddened. Release of occult haemoglobin in vivo from red cell rupture can lead to mucal reddening by release of haemoglobin through the skin (Smith and Ramos, 1976). The observed haemolysis may also be caused by a weakening of red cells increasing the likelihood of rupture on centrifugation.

The response of S. gairdneri Hct values to both pentachlorophenol (PCP) and lindane exposure was quite different to that for

TCIN exposure. The response of an increase in Hct values does not appear to be concentration dependent in PCP exposure, and this could be a function of the variable nature of the stress-response of the Hct increase. The data are consistent with many other studies in which fish are exposed to pesticides (Alabaster and Lloyd, 1982), and confirms that the TCIN induced response is peculiar to that compound. The structures of PCP and lindane are related to that of TCIN (Fig. 4.6) and, therefore, the difference in Hct response must be due to difference in reactivity caused by a characteristic of the structure of TCIN itself, leading to a radically different toxicosis. This will be discussed in later chapters.

It is of interest that Wiersma-Roem et al. (1978) found that exposure of S. gairdneri to dichlobenil (1,3-dichlorobenzonitrile), a compound related to TCIN, for four weeks at 120 - 1280 ug/l caused no changes in Hct values.

Similarly, the Hct exposure to DAC3701 exposure is different from that of TCIN exposure. It is likely on this evidence that the toxicosis of TCIN is related to its electronegativity. DAC3071 has a similar molecular shape to TCIN, its molecular topological index value O_x = 10.59, being not very different from that of TCIN (10.88) (Sabljic, 1982). The effect of chlorine atoms bound to a benzene ring is to increase its electronegativity due to the removal of aromatic electrons from the ring. The replacement of a chlorine atom in TCIN by the electron rich -OH group in DAC3701 would decrease its electronegativity to a certain extent. This change may well contribute to the difference in toxicity and toxicosis observed here, by reducing the toxic interactions with nucleophiles in the body, such as protein residues.

The difference is also possibly related to the ease of metabolism of the toxicant. The metabolic processes and toxic action of DAC3701 in fish would be expected to be similar to those of PCP, as both are phenols of very similar structure (Fig. 4.6). Such phenolic compounds are polar and fish demonstrate a considerable ability to detoxify them by conjugation and excretion (Kobayashi, 1979).

The TCIN-induced reduction in red cell volume may be the prime cause of the interaction between TCIN exposure and the respiratory system, especially since the effect is so rapidly induced. This interaction does not, however, explain the toxic action of TCIN. If a combination of gill damage and diminution in respiratory carrier is

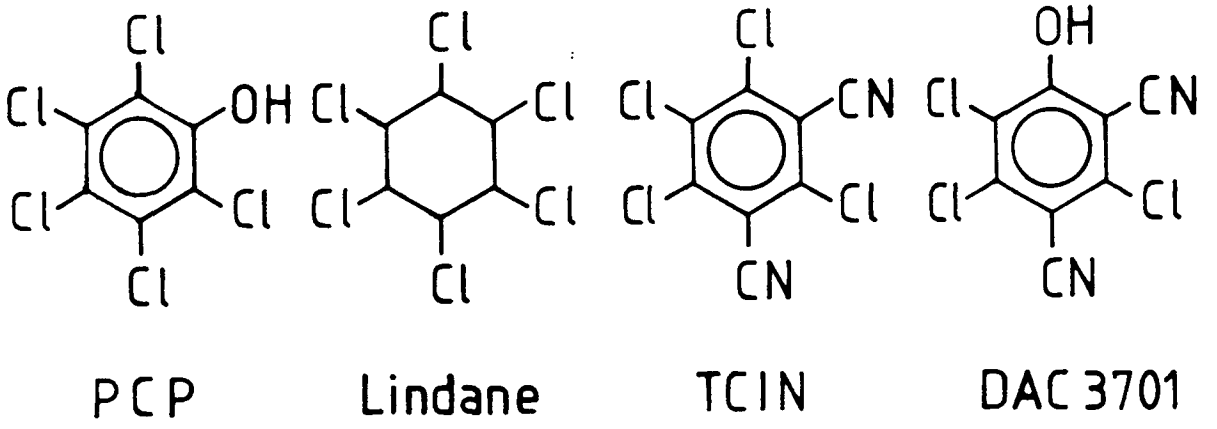


Fig. 4.6 Structures of PCP, lindane, TCIN and DAC3701.

the cause of death by the action of TCIN, local tissue hypoxia would be anticipated. The lack of an increase in tissue lactate levels on acute TCIN exposure negated such a hypothesis. In this context it should be noted that the toxicosis observed by Darrow et al., (1978) on exposure of fish to n-decanohydroxamic acid, a flotation agent, which caused local tissue hypoxia and increases in plasma and tissue lactate, showed two primary differences to that observed here.

Fish exposed to n-decanohydroxamic acid showed the following behavioural toxicosis sequence: excitability, gasping, loss of balance, sporadic bursts of activity and then death. These symptoms are the same as those reported for zinc toxicosis (Affleck, 1952; Skidmore, 1970), which has also been linked to high lactic acid levels (Hodson, 1976) and gill damage (Alabaster and Lloyd, 1982). The toxicosis symptoms observed in fish exposed to TCIN were quite different and as such indicate a different mode of toxic action. Fish exposed to n-decanohydroxamic acid also showed increases in Hct values well over normal levels. Darrow et al. (1978) could not provide a mechanism for the hypoxemic action of n-decanohydroxamic acid in fish since they "showed no differences between control fish and those poisoned" when gill samples were "examined microscopically". It is possible that a morphometric analysis, as used in this study, could have shown significant differences in gill diffusivity which would have been missed by a qualitative examination. It is of particular interest to note that Wiersma-Roem et al. (1978) reported that dichlobenil caused hypertrophy and hyperplasia of gills of S. gairdneri at sublethal levels over four weeks exposure. The concentrations studied were much higher than in this work (120 - 1280 ug/l), but all levels caused slight to pronounced symptoms of gill-damage which "might have serious implications for fish survival at a relatively low oxygen content of the water".

It appears that biliary colour can be used as a diagnostic aid for TCIN exposure in S. gairdneri. Spectral examination of bile did not provide any immediate explanation for the colour change. It is possible that TCIN causes an increase in one or several metabolites occurring in bile, similar to changes caused by starvation. The colour change may also be related to the binding of bilirubin by glutathione S-transferase or ligandin proteins, proteins whose properties in S. gairdneri have been initially described by Nimmo et al. (1979, 1981).

The influence of TCIN exposure on the G S-transferase enzyme group is discussed in Chapter 8. Oxidation of bilirubin glucuronide to biliverdin glucuronide in bile has been suggested as the cause of the same change in biliary colour (Love, 1980). Talbot and Higgins (1982) suggested that the colour darkening may be due to concentration of bile materials by reabsorption of water and salts during bile storage. The sequence of colour changes described by these authors for Salmo salar undergoing starvation was also observed here in starved Salmo gairdneri. It may be that TCIN exposure causes ionic and water imbalance in the blood leading to, as a side effect, a rapid biliary colour change similar to that occurring with biliary storage on starvation.

4.2 BIOCONCENTRATION OF TCIN

4.2.1 INTRODUCTION

The almost ubiquitous finding that pesticides and other organic contaminates accumulate in fish at concentrations well above those in the surrounding medium was an early step in the understanding of xenobiotic processes in the aquatic environment (Jones, 1968; Neely et al., 1974). Bioconcentration is a term generally used in aquatic toxicology to denote the uptake in organisms by direct concentration from water, as distinct from bioaccumulation which involves uptake through the food chain, and which is much harder to identify and assess. The bioconcentration factor (BCF) is defined as the ratio of the concentration of the chemical in the organism to that in the water. It has been the subject of considerable research (Neely et al., 1976; Chiou, 1981). The phenomenon of bioconcentration in fish has been regarded as a partition effect of the pollutant between an organic phase (fish proteins and lipid pool) and an aqueous phase (the surrounding medium). Correlations have been sought, therefore, between the partition coefficient of organic pollutants between an organic solvent similar in properties to a lipid pool (n-octanol being the solvent of choice), and the BCF in fish (Chiou, 1981).

A fish cannot, evidently, be regarded merely as an organic solvent pool. Slow diffusion to the lipid site and metabolic breakdown

delay or possibly prevent equilibrium from being established. There are also a number of lipid compartments of different size and composition within a fish. BCF in fish is generally independent of exposure concentration if equilibrium can be reached. Time to equilibrium varies from species to species, primarily due to the effect of organism size (Reinert, 1972); it also varies with the chemical's solubility (Könemann and van Leeuwen, 1980) and concentration, and the ambient temperature (Matsuo, 1981).

The value of BCF may also depend on the lipid content of the fish's organs (Chiou, 1981). Chiou indicated that the correlation between the octanol-water partition coefficient (Pow) and BCF may depend on the compound's stability. Whilst this is strictly true, the relationship may be modified by the fact that the unchanged compound often accummulates in non-metabolizing, inert lipid pools. Consequently, the relationship between Pow and BCF will depend on the metabolic and lipid status of the animal.

Since BCF is correlatable with Pow, and since Pow depends on the lipophilicity of the compound, a correlation between BCF and water solubility (S) of a compound can be expected. Neely et al. (1974) found that a distinct relationship existed for guppy and S. gairdneri, with a logarithmic regression for S. gairdneri of the form:

$$\log(BCF) = -0.802.\log S' - 0.479 \quad \text{—— Eq. 4.1}$$

with $r = 0.977$ and $N = 7$, where the compounds selected e.g. hexachlorobenzene, p-dichlorobenzene, were structurally similar to TCIN. S' in the above relationship is the solubility S corrected for compound melting (Chiou, 1981).

A correlation of BCF with Pow published by Veith et al. (1979) for a number of fish species, including rainbow trout, is as follows:

$$\log(BCF) = 0.85.\log Pow - 0.70 \quad \text{—— Eq. 4.2}$$

where $r = 0.897$ and $N = 55$.

An investigation of the BCF of TCIN in S. gairdneri was conducted here in order to check if the above considerations also held for the behaviour of TCIN in fish, to see if detectable residues of TCIN exist in S. gairdneri exposed to low TCIN concentrations, and to

observe the time-course of concentration. Analyses of fish suspected of being TCIN stressed or poisoned at the Bridport trout farm had showed no detectable residues (Chapter 2). Rapid metabolism of TCIN may cause the decline of tissue levels below detectable limits on a single-event exposure. A test was needed to observe if any appreciable bioaccummulation of TCIN does occur in order to gauge the efficacy of depending on measures of tissue TCIN levels for diagnosis of exposure. It was also decided to observe which organs concentrate TCIN residues to high levels, and to determine if levels of TCIN build up in exposed fish to levels that may be toxic for human consumption. This was regarded as highly unlikely given the low oral toxicity of TCIN to mammals (F.A.O., 1975).

4.2.2 MATERIALS AND METHODS

Partition coefficient

C^{14}-TCIN, synthesized as detailed in Chapter 5 (activity 6067 dpm/ug) 0.7 mg, and TCIN, 20 mg, was stirred between 2 ml redistilled n-octanol and 2 ml 0.01 M phosphate buffer (pH 7.0) for 48 h at 20° C. The two layers were then filtered (glass-fibre filter) and aliquots radiocounted. The test was performed in triplicate. Attempts at measuring Pow by reverse-phase TLC on n-octanol saturated silica gel by the method of Boyce and Milborrow (1965) were abandoned due to the low resolution of Rf values.

Exposure of fish

A group of 34 S. gairdneri (8-14 g) were exposed to 10 ug/l TCIN over 21 days in the flow-through apparatus described in 3.1.2, with daily feeding to satiation, in a 40 l tank with 2 l/min flow rate, at 14-16° C. Five fish were removed at each of 2, 4, 8, 12, 16 and 24 days. At 24 days the remainder of the fish were removed for organ analysis. Fish were killed in 0.2% w/v tricaine methanosulfonate and stored at -18° C until analysis. S. gairdneri, Galaxias maculatus and G. truttaceus which had died of TCIN poisoning in LC50 experiments at concentrations of 35 - 40 and 20 - 25 ug/l were also analysed for whole-body TCIN concentrations. These animals had been frozen within

2 h of death. Also, five each of G. maculatus and G. truttaceus which had been exposed to 8 and 13 ug/l for 96 h were killed, frozen and subsequently analysed for whole-body TCIN concentrations.

Extraction and analysis

Whole fish or pooled organs were homogenised immediately after rapid thawing in a Sorvall Omni-mix blender. A 2 g aliquot was taken and homogenised with 5 ml acetonitrile for 4 min at high speed. The mixture was then rehomogenised after addition of 25 ml acetone for 4 min at high speed. The whole was passed through a cotton-wool plug with two washings of acetone (10 ml) into an extraction funnel containing 100 ml 2% sodium sulphate solution. After mixing, 25 ml redistilled hexane was added and the funnel was shaken vigorously by hand for 1 - 1.25 min. The aqueous layer was discarded, and the organic layer was washed with 50 ml 2% sodium sulphate solution. After discarding the aqueous layer, the hexane layer was dried (anhydrous sodium sulphate), evaporated to $\leqslant$ 2 ml and washed through an alumina column (2 g Al_2O_3, deactivated) to remove fat. The final solution was analyzed by G.C. as detailed in 2.1.2. Liver, fat and caecae and muscle tissue were separately analysed from S. gairdneri exposed to 10 ug/l for 21 days. Mature testes and eggs were also analysed from G. maculatus exposed to 12 ug/l for 96 h.

4.2.3 RESULTS

24 day whole body TCIN concentrations and BCF values for S. gairdneri exposed to 10 ug/l TCIN are shown in Table 4.7. No TCIN residues were detectable in extracts of S. gairdneri exposed to 10 ug/l TCIN for less than 24 days. Individual organ extracts of S. gairdneri from the 24 day exposure also contained TCIN levels below the G.C. detection limit of 0.01 mg/kg. 24 day exposed whole fish extracted by an exhaustive method using water - H_2SO_4 - acetone, with hexane - isopropyl ether secondary extraction, showed similar results to those extracted by the MeCN - acetone homogenization method described above.

No residues were detectable in S. gairdneri, G. maculatus and G. truttaceus individuals exposed to higher levels of TCIN over 96 h.

Table 4.7 Bioconcentration of TCIN in S. gairdneri exposed to 10 ug/l over 24 days

Exposure time (days)	Proportion with residues	Residues (ug/kg)	BCF *
24	3/5	100,200,100	13.3
2, 4, 8, 10, 16	0/5	< 10	< 1.0

* in fish where residues are detected.

Similarly, no residues were detectable in individuals of the above three species which had died of TCIN exposure during the LC50 experiments. These results indicate that death by aquatic TCIN exposure is not related to high tissue levels of the parent compound. Tissue levels of TCIN attained in whole fish were not deemed at all harmful for human consumption by F.A.O. (1975) guidelines. Log Pow was determined as 4.38 ± 0.12.

4.2.4 DISCUSSION

Equations 4.1 and 4.2 can be used to see if TCIN follows the behaviour of other compounds in the same class in its bioconcentration in S. gairdneri. The solubility of TCIN in water is 0.60 mg/l. The value of S', S corrected for energy of melting, is given by the relationships:

$$\log (fs/_{fi}) = -K.(Tm - 298.15)/2.303 \text{ —— Eq. 4.3}$$

where K is an empirical constant of magnitude 0.02273, corresponding to an averaged entropy of fusion Hf/Tm of 13.46 (Chiou, 1981), and

$$\log S' = \log S + \log(fs/_{fi}) \text{ —— Eq. 4.4}$$

The correction is necessary due to the effect of the entropy of melting on solubility values. Both S and S' are in mole/l. The melting point of TCIN is 250° C, and equations 4.3 and 4.4 give log S' = -5.1813. Equation 4.1 gives a BCF value of 4,747. From the value of log Pow for TCIN determined here, equation 4.2 gives a BCF value of 975 in S. gairdneri.

The value of BCF measured here for TCIN in S. gairdneri at 24 days exposure is only 13.3 and this does not agree with the two estimates predicted above. Since TCIN does not apparently reach an equilibrium concentration, or plateau phase, in the body concentration by 24 days (Table 4.7), it, therefore, appears that TCIN does not behave in the same way as other organic pollutants in terms of its bioaccumulation, and that its metabolism is too rapid to prevent equilibration occurring.

The physical behaviour of TCIN does follow the general trend observed for compounds as regards the relationship between Pow and S. Chiou (1981) gives the following regression:

$$\log Pow = -0.862.\log S + 0.710 \quad \text{Eq. 4.5}$$

where r = 0.994 and N = 36, extending over 5 and 6 orders of magnitude in Pow and S, respectively. This predicts a log Pow value of 4.97, agreeing fairly well with that obtained here.

It appears that bioconcentration of TCIN does occur, but only to a limited extent and over a long time period, 24 days not being long enough for equilibration to occur. In this, TCIN does not follow the behaviour of other related chlorobenzenoid compounds (Neely et al., 1974). It is apparent, therefore, that TCIN is rapidly metabolized in the body, both on lethal and sublethal exposure. This indicates either that the species investigated have an efficient detoxication or excretion system for disposition of TCIN, or that TCIN binds rapidly to cellular components.

These results also indicate that, although no TCIN residues were detectable in S. gairdneri from the Bridport trout-farm collected during stress periods suspected to be TCIN - induced, this does not discount possible TCIN toxicosis. More intensive field-work, not possible in this study, is needed in order to correlate observed stress periods with water TCIN concentrations.

Since TCIN is rapidly metabolized, but is also highly toxic, it was decided to investigate the course of its metabolism in an attempt to elucidate the toxic action of the compound. This work is reported in the following chapters.

CHAPTER 5

SYNTHESIS OF (C^{14} - CN) CHLOROTHALONIL

5.1.1 INTRODUCTION

A number of methods are available for the study of the metabolism of an organic compound in an organism. They include separative techniques such as thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), solvent extraction and column chromatography. In order to identify the route of metabolism, characteristic properties of the compound moiety or its suspected metabolites must be exploited. Typical "tags" for such studies include characteristic ultraviolet (UV) spectra, fluorescence spectra, characteristic colour reactions and radiolabels.

Several methods of tracing TCIN in metabolic studies were investigated. Specific TLC reagents do not exist for the TCIN moiety, which is particularly unreactive. Mallet and Frei (1971) indicated a possible method for the visualization of pesticides on TLC but they found it to be of no use for detection of TCIN. The UV spectra of TCIN and its principal fungal metabolite, a mono-glutathione conjugate, are shown in Fig. 5.1. Peak maxima are not sufficiently different to enable spectrophotometric differentiation. Peak maxima also occur in a spectral region which is not suitable for unique identification of the TCIN moiety, as many other biological materials have significant absorbance in this region. The ϵ values are not sufficiently high to allow detection of levels of TCIN likely to occur in metabolism studies. A solution of 20 ug/l TCIN would give an absorbance value of only 0.005 units/cm.

In fluorescence spectroscopy, irradiation of a compound at a characteristic wavelength, the maximum of the excitation spectrum, produces a reradiated emission with a spectrum with an emmission wavelength maximum characteristic of that compound. The procedure allows a "double-check" on the component analysed due to the combination of two characteristic wavelengths. It has great sensitivity as an analytical technique especially with strongly fluorescent compounds such as Rhodamine B which can be detected down to 0.1 ug/l.

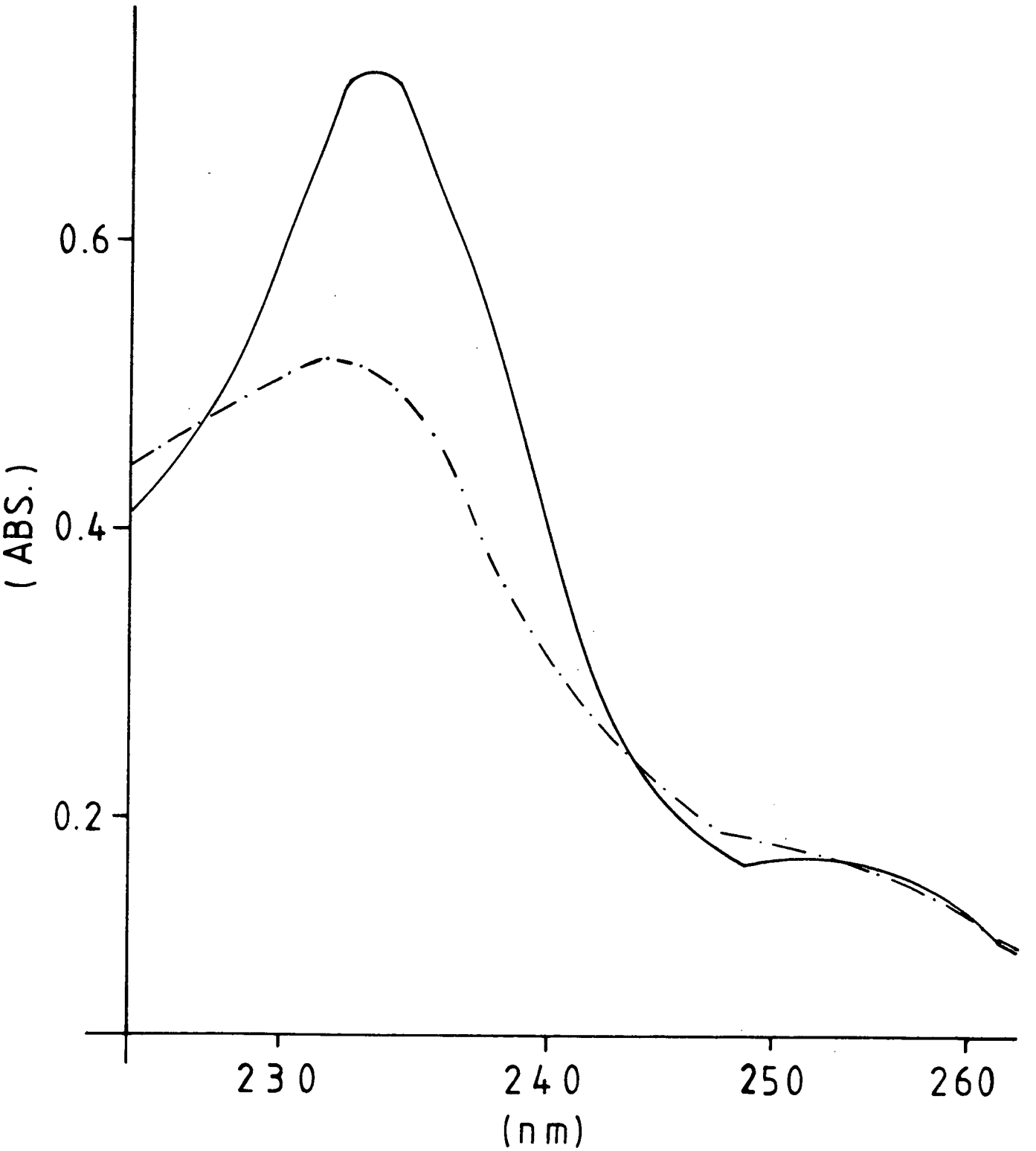


Fig. 5.1 Ultraviolet spectra of TCIN (——), and the monoglutathione conjugate, G1 (–.–) (MeOH). ϵ = 62.1 units mM/cm (TCIN); 22.01 units/mM/cm(G1).

The excitation and emission spectra for TCIN and the phenolic degradation product, DAC3701, are shown in Fig. 5.2. A plot of mission at 440 nm on irradiation at 390 nm for TCIN against concentration is shown in Fig. 5.3, along with the change of peak wavelength and peak form. A number of limitations put constraints on the usefulness of this approach. The emission spectrum of TCIN changes wavelength and is diffuse in profile at low concentrations. The minimum concentrations at which this technique is effective for detection of TCIN are in the range 20 - 50 ug/l, not low enough for the concentration ranges expected in metabolic studies of fish exposed to TCIN in the range 5 - 20 ug/l. The spectral characteristics of TCIN and DAC3701 do not differ substantially enough to enable clear differentiation of these compounds. Therefore, fluorescence spectroscopy is not a suitable technique for a study of the metabolism of low levels of TCIN and its metabolites.

Phosphorimetry of TCIN is discussed by Zander and Hutzinger (1971), and compared to fluorescence analysis. Its sensitivity as regards TCIN itself appeared to be sufficient for metabolic studies, but the technique was not further investigated in this study.

It was decided to investigate the possibility of radiolabelling TCIN. Radiolabelling of a xenobiotic enables tracing and quantifying the parent or its metabolites after separation, with a high level of sensitivity. This sensitivity depends on a number of factors, including the strength of the radioactivity incorporated into the compound. The upper limit of label strength is set by the level at which the radioactivity itself becomes harmful to the organism (especially in long-term exposures), and the experimenter. It is also set by the availability and expense of the radioisotope. The lower limit is set by the resultant accuracy of the final count compared to the background, and is a function of the label concentration in the xenobiotic, the concentration of the labelled xenobiotic moiety in the sample and the sample size.

The degree of chemical "stability" of the isotopic centre in relation to the host molecule, is of vital importance in metabolic studies. If the label is at a site which is intimately linked with the compound moiety in question, and is not labile, then it is suitable for tracing studies. The half-life and radiation energy of the isotope

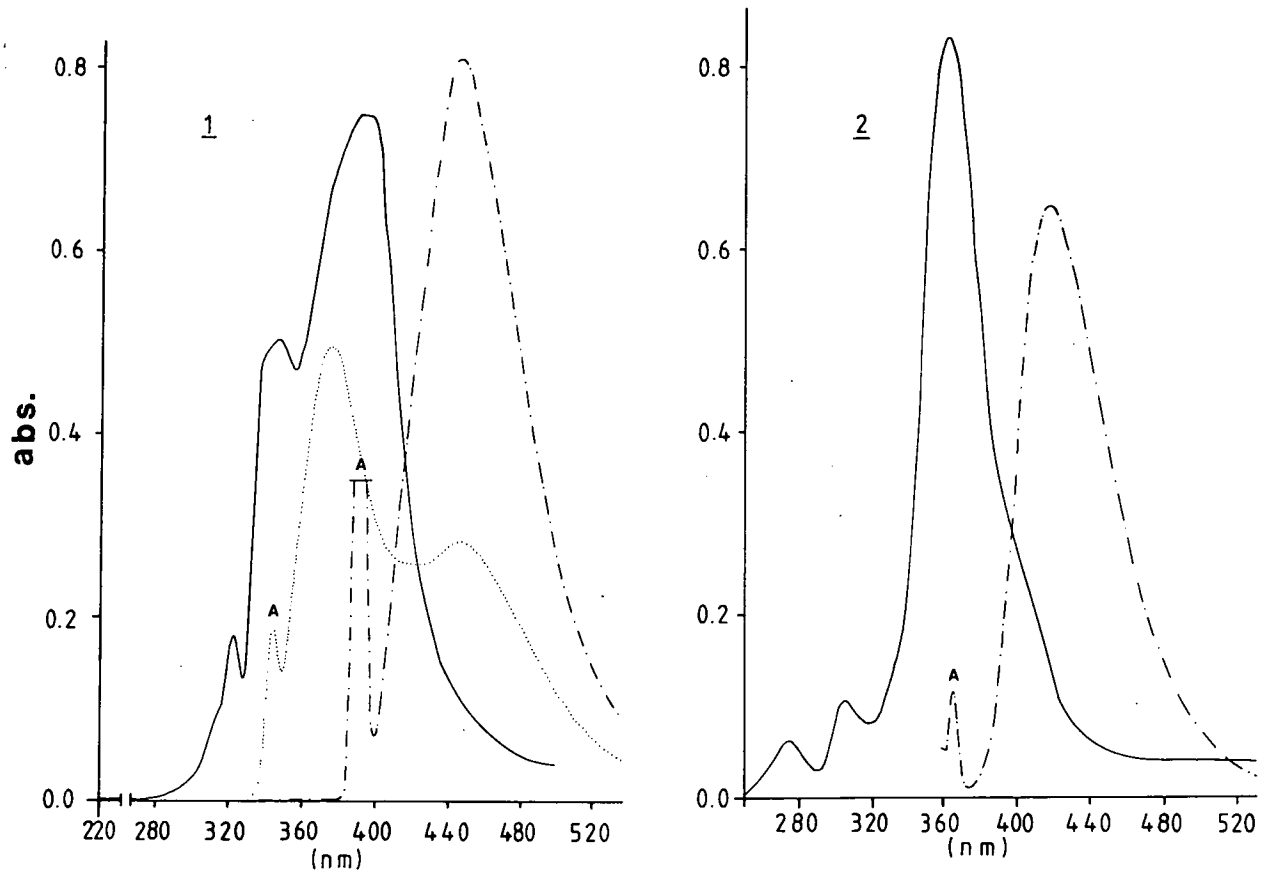


Fig. 5.2

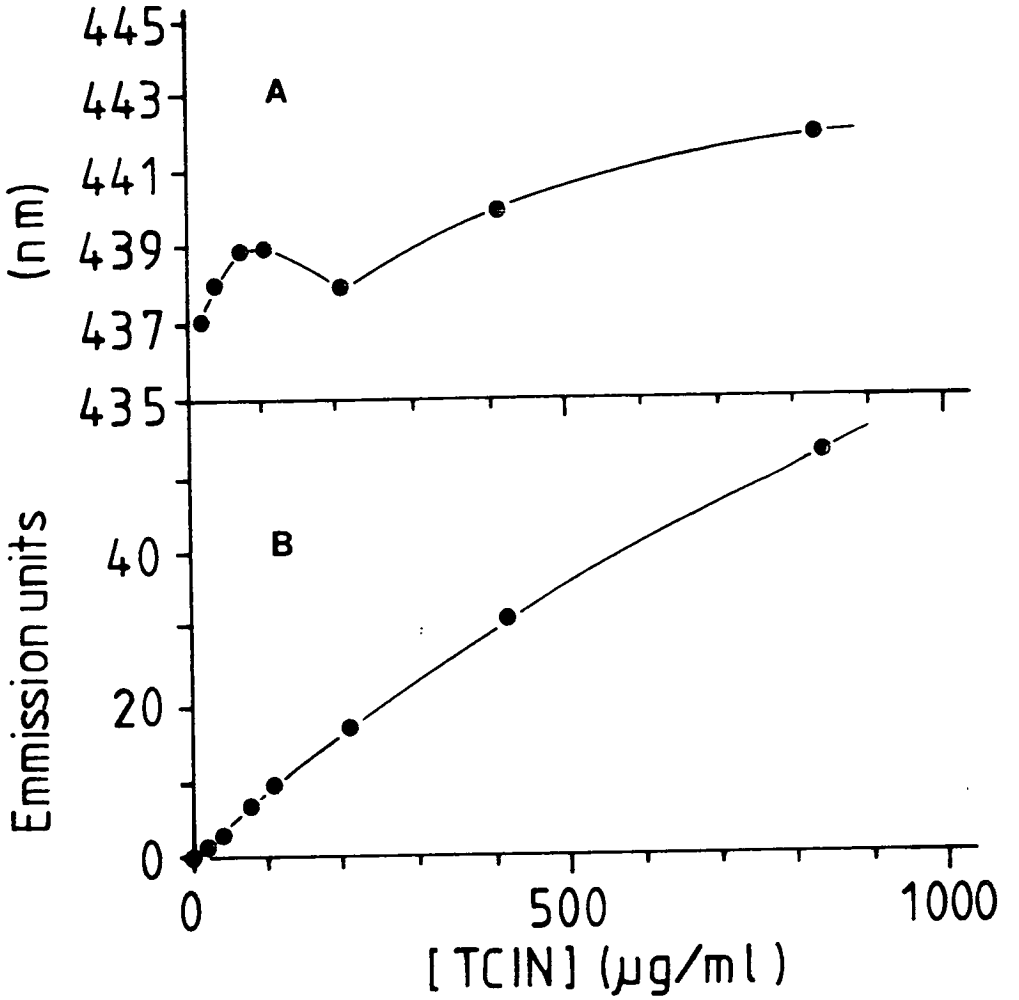
1: Excitation and emission spectra for TCIN(EtOH).
(——): excitation spectrum; (...): emission spectrum, irradiation at 344 nm; (—.—): emission spectrum, irradiation at 390 nm. A = scatter at irradiation wavelength.

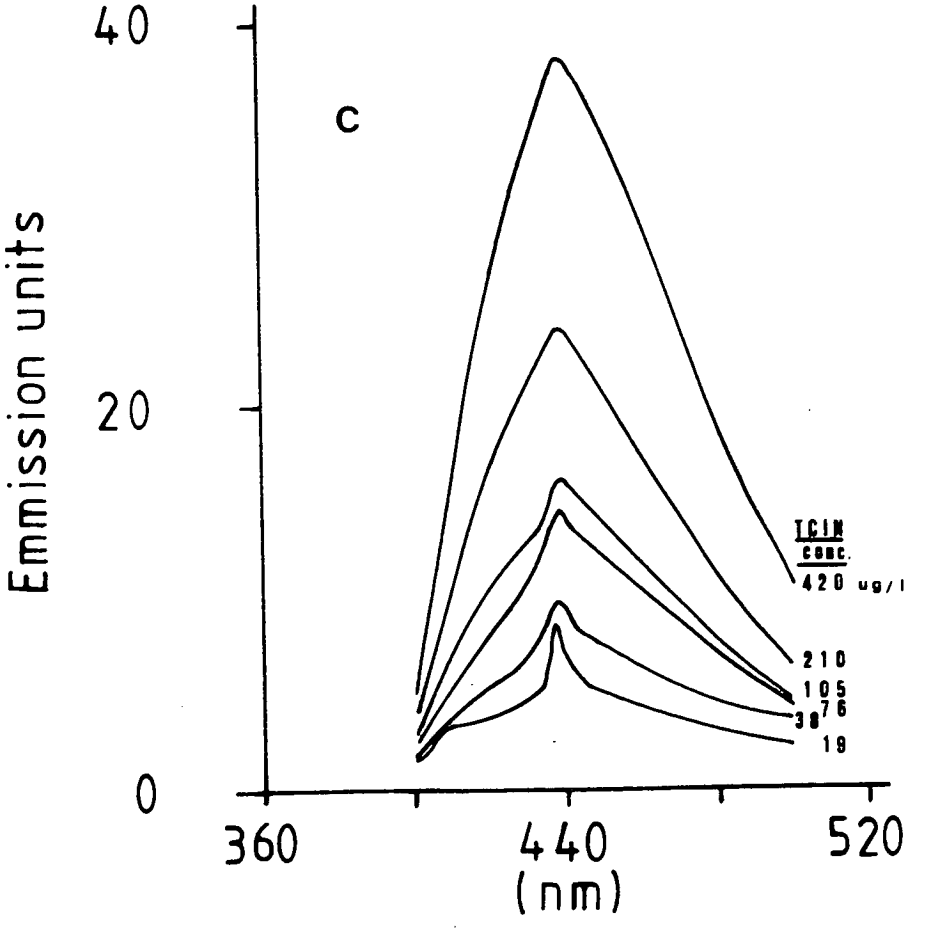
2: Excitation and emission spectra for DAC3701(EtOH).
(——): excitation spectrum; (—.—): emission spectrum, irradiation at 365 nm. A = scatter at irradiation wavelength.

Fig. 5.3 Fluorescence spectroscopy of TCIN solutions.

A, B = variation in wavelength and emission of peak solutions (EtOH), excited at 390 nm.

C = emission peak profiles of TCIN solutions (EtOH), excited at 390 nm.


445
443
441
439
437
435
(nm)
A
B
40
20
0
Emmission units
0
500
1000
[TCIN] (µg/ml)


40
20
0
Emmission units
C
TCIN conc.
420 ug/l
210
105
76
38
19
360
440
520
(nm)

selected are also crucial to the tracing study. Limitations are also encountered in the area of chemical separation procedures of different metabolic species.

Radiolabelled TCIN had been used in previous studies on the compound's biological fate (Long and Siegel, 1975; Szalkowski and Stallard, 1976; Chin et al., 1981). The material was supplied to those researchers by the manufacturers of the fungicide, Diamond Shamrock Chemical Corp., and was ring-labelled. This source of supply was cut-off in 1981, apparently due to public concerns voiced about the carcinogenicity of TCIN (Siegel, pers. comm.). Consequently, a synthetic approach was needed.

On considering the synthesis of a radiolabelled compound, the primary concern is the efficiency of the process with regard to overall isotopic yield. Each synthetic step involving the radiolabel will inevitably involve some loss, by chemical or technical means. The most effective synthetic design is that in which reaction yields are high and the number of steps involving labelled reactants is as low as possible.

In the case of TCIN, the choice of isotope is clear. There are no suitable nitrogen isotopes. Cl^{36} is a beta emitter with a long half life. However, labelling of the Cl positions is likely to be of low isotopic yield, as industrially it is performed in the gas phase. Also, since the chlorine groups are considered to be the biochemically labile functions (Vincent and Sisler, 1968; Tillmann et al., 1973), it would be inappropriate to use this isotope as a tracing label. There are, therefore, only two possibilities for radiolabelling TCIN, both involving carbon-14. A labelled benzenoid precursor could be used to produce ring-labelled TCIN, or the CN functions could be used as the labelling site.

The industrial method of producing TCIN is by a continuous vapour-phase reaction sequence involving the ammoxidation of meta-xylene followed by the reduction of the diamide formed and its vapour phase chlorination with an overall purity of $\geqslant 95\%$ (U.S. patent, 1972). This procedure was attempted unsuccessfully on a laboratory scale by J.R.Siegel (pers. comm.), after supply of C^{14}-TCIN was cut-off. In order to make C^{14}-TCIN by this procedure, however, either ring-labelled or methyl-labelled meta-xylene would be needed as a starting material since it could not conveniently be made by laboratory

synthetic methods without considerable expense. On receiving a quote from Amersham Pty. Ltd. of $10,000 for a custom synthesis of this starting material, and considering the difficulties of performing a continuous reaction sequence in the laboratory, this approach was abandoned.

The only other possibility was to attempt to incorporate carbon-14 into the nitrile functions of TCIN. It appears in previous studies that the TCIN cyano groups are chemically and biologically stable. The benzonitrile moiety is strongly aromatic with π bonding between the benzene ring and CN allowing delocalization of electrons and considerably reducing the possibility of attack at the CN positions under all but the harshest conditions. Hydrolysis of the nitrile to benzamide has been reported by Szalkowski and Stallard (1976), but this process does not involve loss of the carbon.

A number of synthetic routes presented themselves. Introduction of the label as C^{14}-CN by Sandmeyer replacement of a diazonium group is illustrated in Fig. 5.4. Replacement of the two amine functions by nitrile could be performed at the same time, as illustrated, or sequentially. The advantage of this route is that the label is introduced at the final synthetic step.

A second synthetic route is illustrated in Fig. 5.5. It involves the introduction of the radiolabel early in the sequence via a Grignard reaction, followed by amidation and dehydration, a process reported for the industrial production of phthalonitriles in a United States patent (1966). The disadvantages include likely low isotopic yield in the Grignard reaction, and subsequent low yields.

A third possible means of synthesizing (C^{14}- CN) TCIN is shown in Fig. 5.6. It involves di-bromine replacement with C^{14}- CN from C^{14}-CuCN in the tetrachlorinated compound.

A synthetic route related to the latter is illustrated in Fig. 5.7. Replacement of a meta-halogen in the benzonitrile by C^{14}- CuCN is followed by vapour-phase chlorination. Meta-iodobenzonitrile can be synthesized by the route shown in Fig. 5.8.

The routes illustrated in Figs. 5.4, 5.6 and 5.7 were attempted with an unlabelled nitrile source in order to assess the viability of each route.

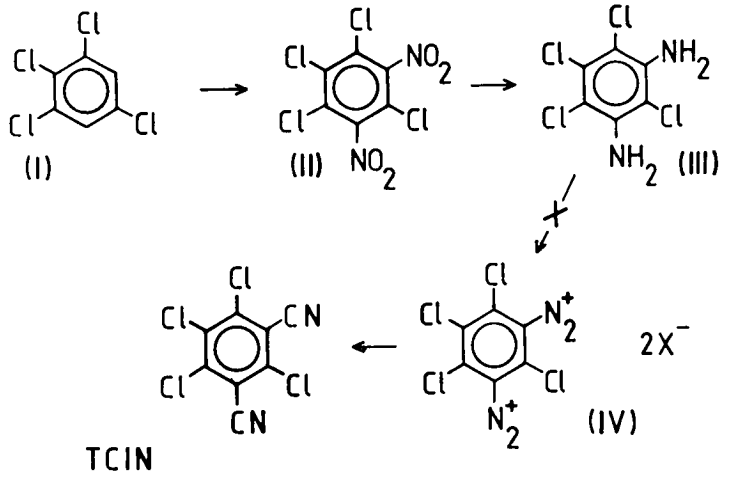


Fig. 5.4 Route 1. Synthesis of TCIN by replacement of diamino in (III) by CN by Sandmeyer reaction.

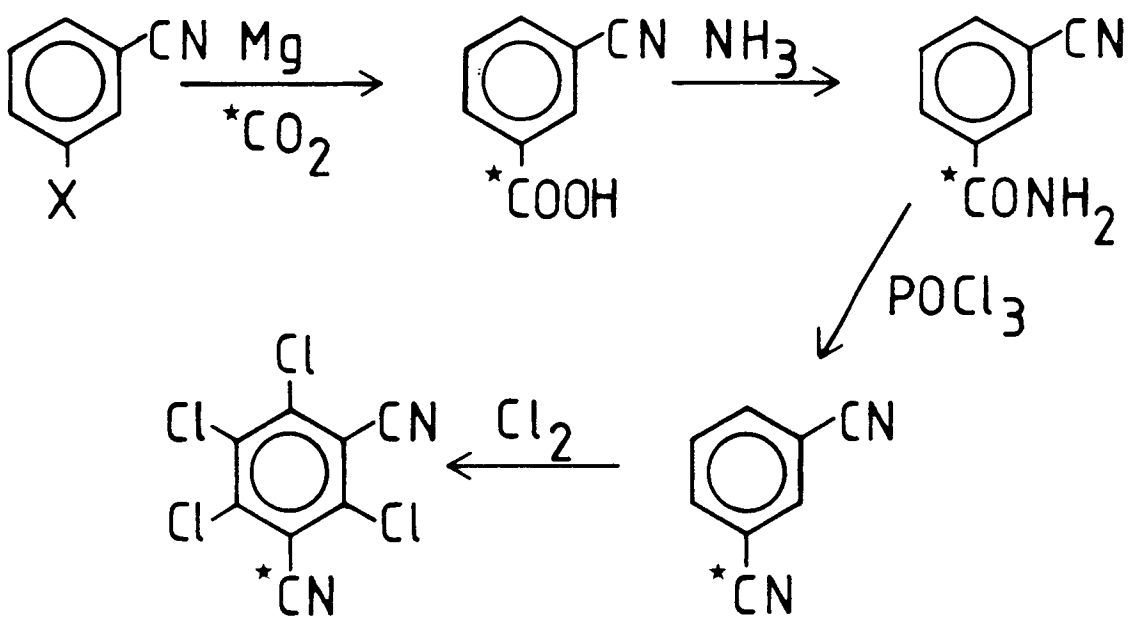


Fig. 5.5 Proposed benzamide route for the synthesis of (C^{14}-CN)TCIN.
C^{14} atoms are marked with (*).

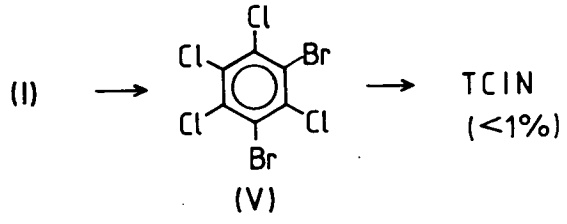


Fig. 5.6 Route 3. Synthesis of TCIN by simultaneous replacement of bromines in (V) by CuCN.

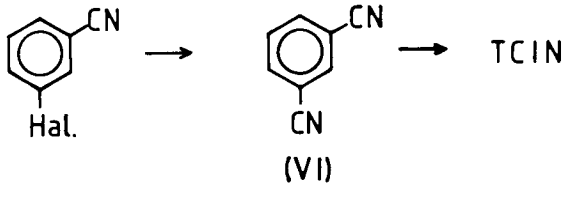


Fig. 5.7 Route 4. Synthesis of TCIN by replacement of halogen in m-halobenzonitrile to give (VI), then TCIN by chlorination.

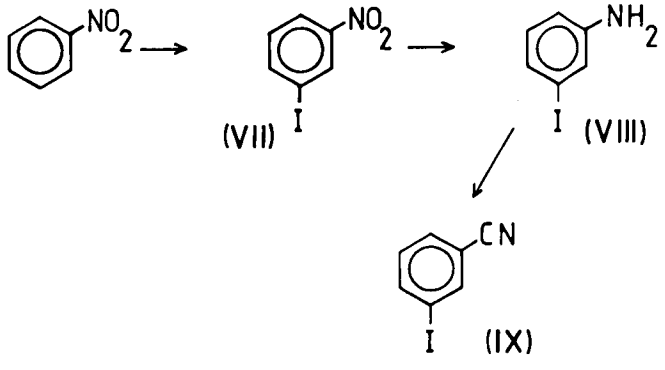


Fig. 5.8 Synthesis of m-iodobenzonitrile (IX).

5.1.2 MATERIALS AND METHODS

C^{14}-KCN (57.81 mCi/mmol) was obtained from Amersham International, Australia. NMP and DMF were freshly distilled and dried. Melting points were determined on a Yanagimoto Seisakusho micro-melting point apparatus, and were uncorrected. Preparative TLC was performed on Merck silica gel GF254. Infrared spectra were determined as mulls on a Beckman IR-33 spectrometer. N.m.r. (H) spectra were performed at 1000 MHz with a Jeol JNM-4H-100 spectrometer, tetramethylsilane as internal standard. E.I. mass spectra were obtained on a Micromass 7070F double focussing mass spectrometer. G.C. analysis was performed on a Philips PV4000 series gas chromatograph, at conditions indicated, with an SE30 column, nitrogen gas as carrier.

Synthesis details

(III) 2,4,5,6-tetrachlorodiaminobenzene

Produced by nitration of 1,3,4,5-tetrachlorobenzene, by the method of Jackson et al. (1902) with chloroform extraction and recrystallization from Lt. petrol (40-60°); 70% yield. Quantitative reduction of the dinitro compound gave (III), by the method of Yakobson et al. (1963).

(IV) Diazonium salt of (III)

Attempts at diazotisation by the method of Hodgson and Heyworth (1949), and at making the fluoroborate salt under anhydrous conditions by the method of Doyle and Bryker (1979) were unsuccessful.

(V) 1,3-dibromotetrachlorobenzene

Synthesized in 90% yield by the method of Hugel et al. (1954), as fine colourless needles, M.P. = 250-253° C.

Cyanation of (V)

(V) was refluxed in dry DMF with fresh CuCN (1.15 molar ratio). Samples were taken at intervals over 4 h and worked up according to Friedman and Schechter (1961), Method C. The dried benzene layers were analyzed by G.C. (210° C), as in Fig. 5.9.

(VIII) m-iodoaniline

Nitrobenzene was iodinated according to Barker and Waters (1952), to give a 47% yield of an oil, 90% pure by G.C., (VIII). Reduction by the method of Yakobson et al. (1963) gave (VIII), as an oil, pure by G.C., in 80% yield.

(IX) 3-iodobenzonitrile

(VIII) was subjected to a Sandmeyer reaction (Hodgson and Heyworth, 1949) to give 41% (IX) as colourless needles (M.P. = 39-40° C, lit. 40° C), after preparative TLC (Rf 0.54, Lt. petrol-acetone, 8:1).

(VI) Isophthalonitrile

(IX), 229 mg, was dried in vacuo and dissolved in dry distilled NMP (7 ml). CuCN (produced by method of Chabannes et al. (1967), 90% yield, and dried by azeotropic benzene distillation in vacuo), 104 mg was added and the whole refluxed under nitrogen for 2.5 h. The mixture was cooled, mixed with hot 30% NaCN solution, diluted to 100 ml with cold water, and extracted with benzene (3x20 ml). The benzene layers were combined, extracted with 10% NaCN (2x50 ml), water (3x50 ml) and then dried (Na_2SO_4, anhyd.). The benzene was evaporated to give 150 mg crude material which was subjected to TLC (lt. petrol-acetone, 8 : 1.5). This gave 118 mg (VI) (92% yield). M.P. = 162 - 164° C (lit. 162° C). M^+ = 128. N.m.r. ($CDCl_3$), 7.6 - 8.1, multiplet.

(VI) can be purified by sublimation in vacuo, at 100-150° C. The above reaction was performed with C^{14}-KCN, diluted to 1.18% by KCN, to produce C^{14}-CuCN (90% yield) and (VI), (C^{14}-CN) in 75% yield.

Tetrachloroisophthalonitrile

(VI), 100 mg, was dried in vacuo and introduced into a 100 ml thick wall, round flask with a Rotaflo (R) tap adaptor, with 400 mg of preactivated charcoal as catalyst.

Fine charcoal was preactivated by heating in the above bulb filled with chlorine gas at 220° C for 2 h. After evacuation and refilling with chlorine the bulb was sealed and heated at 250-300° C by immersion in a Woods metal bath for 4.5 h. Cooling, evacuation, acetone extraction (3x20 ml) gave, on filtering, evaporation and

vacuum drying, 160 mg of TCIN (95%), (75% yield). G.C. analysis was performed on cooled flask wall deposits during a trial run (Fig. 5.10).

The same yield was obtained on performing the reaction with (C^{14}- CN) from the previous reaction. Radioactivity = 6065-6300 dpm/ug; expected = 5830 dpm/ug. TCIN produced by this method, was identical by M.P., TLC, G.C. and M.S. with purified technical material.

5.1.3 DISCUSSION

Several routes were attempted. Route 1 (Fig. 5.4) was unsuccessful due to the failure of formation of the bis-diazonium salt(IV). Hodgson and Heyworth (1949) successfully generated isophthalonitrile by the analogous route, but at a yield of only 25%. This was repeated and gave similar but variable yields. Chlorination of sites adjacent to the amine functions appears to inhibit formation of a stable bis-diazonium salt, although the high yield of bis-diazonium salt (82%) generated from 4,6-diamino-m-xylene and a subsequent Sandmeyer generation of the di-cyano compound (no yield given) seems to preclude a direct steric effect (Ruggli and Caspar, 1935). This lack of success may be attributed to the presence of the highly deactivating chlorines which reduce the basicity of the amine functions and reducing the stability of the bis-diazonium salt. Secondary chlorination of the isophthalonitrile formed by the di-Sandmeyer reaction on 1,3-diaminobenzene (Hodgson and Heyworth, 1949), is a possible alternative but low reaction yield and consequent radiolabel loss prohibit its use.

Route 3 (Fig. 5.6) involved an attempt to replace Br with CN by direct nucleophilic replacement, using CuCN, in 1,3-dibromotetrachlorobenzene (V). The cyanation reaction was monitored by gas chromotography (Fig. 5.9), and showed significant production of the primary replacement product (Fig. 5.9, 5a). However, it appears that the second bromine no longer competes successfully with the remaining chlorines for replacement and may be aiding multiple substitution by "solvation" effects. A number of products is observed, probably with multiple replacement, and only a small fraction of the total is TCIN (<1% at 4 h).

Route 4 (Fig. 5.7) involved replacing halogen (Cl, Br, I) with CN from CuCN in 3-halogenated benzonitrile, and chlorinating in the vapour phase. The first step was successfully carried out for Br and I, with I giving the highest yields (Table 5.1). Reaction conditions were investigated (Newman and Boden, 1961; Friedman and Schechter, 1961), and both N-methyl pyrrolidone (NMP) and dimethylformamide (DMF) were used as solvents. Catalysis of this reaction type has been reported (Cassar et al., 1974, 1979) but was not investigated.

The use of the copper (I) salt in nucleophilic replacement is a reaction much influenced by choice of solvent and temperature (Bacon and Hill, 1964; Friedman and Schechter, 1961; Newman and Bowden, 1961). Reaction rates vary by a factor of 200 depending on the solvent. The ease of entry of the substituent nucleophile into the host molecule from its copper (I) salt is in the order : $Cl > Br > I > CN$, $SPh > SCN$. The mechanism involves a tetrahedral copper (I) complex with π-bound solvent ligands. The greater difficulty of replacement by CN,SPh and SCN is due to the higher stability of the copper complex. Similarly, the competitive ability of the solvents for ligand sites influences the reaction rate, although the liganding ability of the solvent is crucial to the reaction (Bacon and Hill, 1964). NMP and DMF have been found to be optimal solvents for this reaction, and this is born out in this work.

It is well known that aryl nitrile functions are meta-directing and deactivating for general nucleophilic substitution reactions with activated aryl halides. However, para and meta substitution does not readily affect the cuprous salt substitutions, and the order of halogen displacement from the aryl nucleus is I, $Br > Cl \gg F$, in contrast to that of the general nucleophilic substitution reaction which is $F > Cl \simeq Br \simeq I$. The yields of the reactions shown in Table 5.1 agree with this argument.

Ortho-substitution in the Cu(I) reactions is promoted by a "built-in solvation" effect (Bacon and Hill, 1964). This effect was used by Koopman (1961) in the synthesis of the compound 2,6-dichlorobenzonitrile (dichlobenil), related to TCIN (Fig. 5.11). In using the cuprous cyanide replacement of chlorine, he was able to selectively replace the chlorine ortho to the nitro function due to the "solvation" effect of the latter group. Such an approach was not available or warranted in the synthesis of TCIN. It is of interest to note that in

TABLE 5.1 CN replacement yields in the reaction between CuCN and m-halobenzonitrile

Solvent	Halogen		
	Cl	Br	I
DMF	N.R.	50%	-
NMP	-	65%	92%

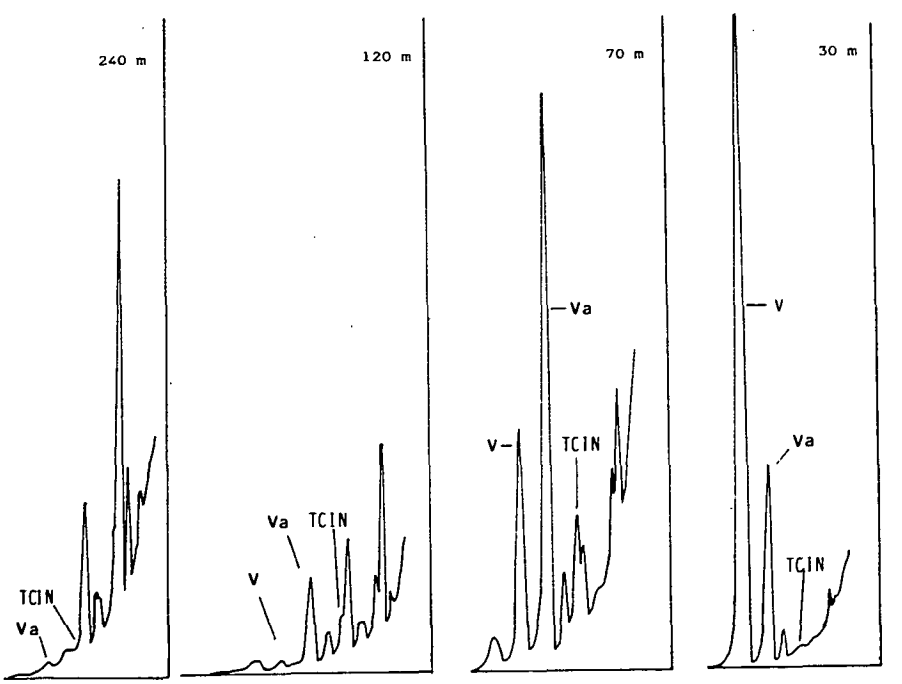


Fig. 5.9 Gas chromatograms of the reaction between CuCN and 1,3-dibromo-tetrachloro-benzene (V) in refluxing DMF at different reaction times (min). Va is the mono replacement product.

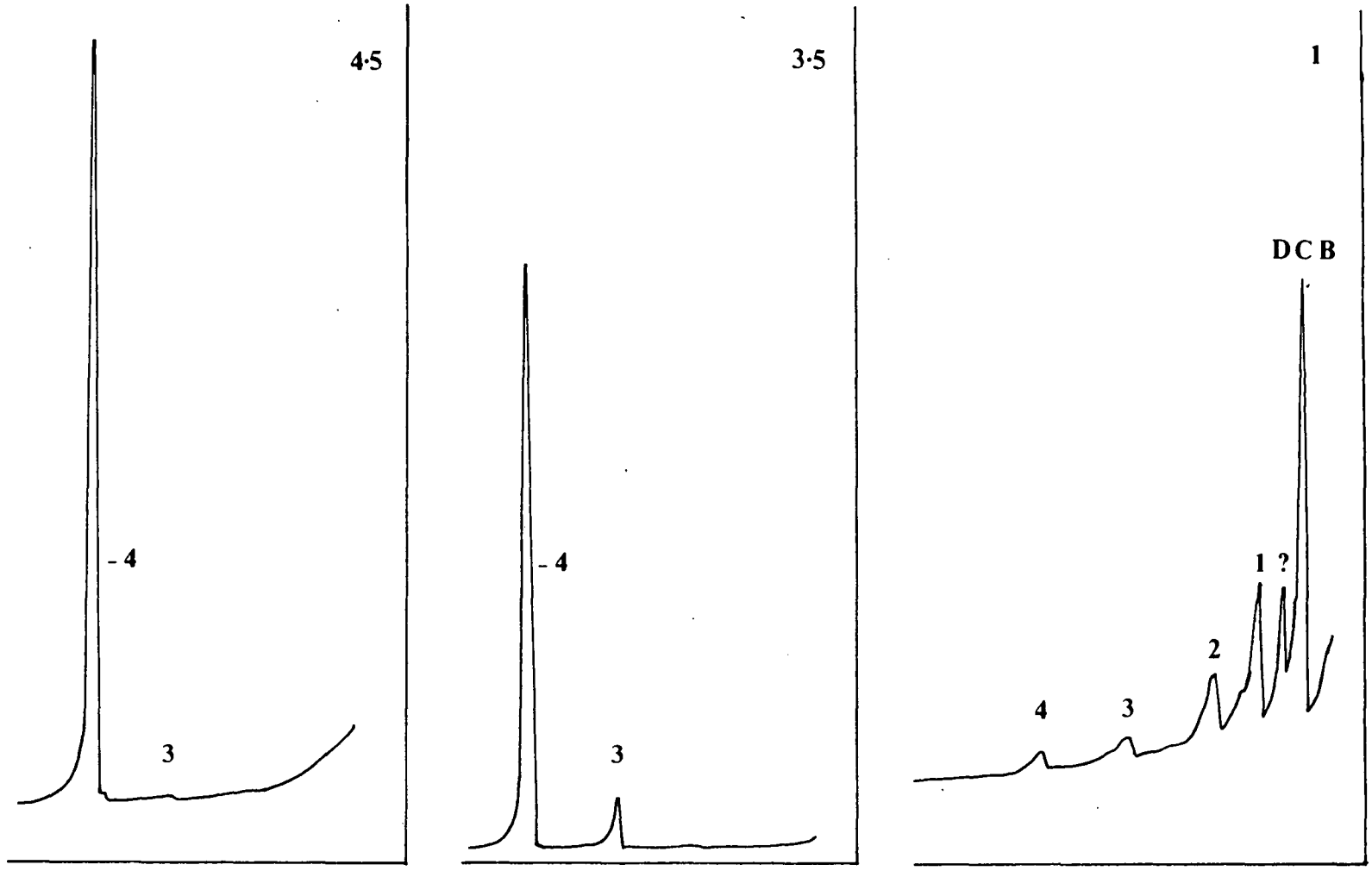
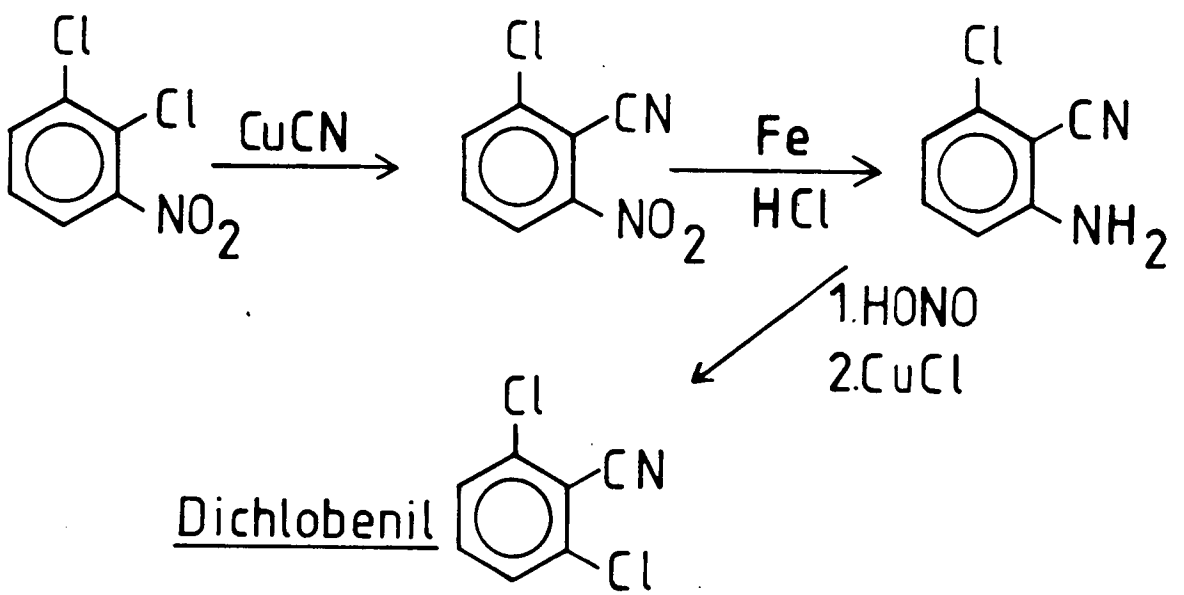


Fig. 5.10 Gas chromatograms of vapour phase chlorination mixtures of isophthalonitrile (DCB, VI) at different reaction times (h), at 250 - 300° C.
1,2,3,4 = mono, di, tri and tetra (TCIN) chlorinated products.



5.11 Synthesis of dichlobenil (Koopman et al., 1961).

the Sandmeyer conversion of the amine group to chlorine, Koopman experienced difficulties in the diazotisation due to the low basicity of the amine.

Vapour-phase chlorination gave a gradual conversion of isophthalonitrile (VI) to TCIN at temperatures between 250-300° C with high yields, although some form of catalysis is important (Japan patent, 1968). Attempts at chlorination in high boiling solvents were unsuccessful. The gradual conversion of 1,3-benzodinitrile to TCIN by sequential addition of chlorine in the vapour phase can be seen in the gas chromatograms illustrated in Fig. 5.10.

Synthesis of 3-iodobenzonitrile (IX) was as indicated in Fig. 5.8. CuCN was made fresh and dried in vacuo. After optimization, the reaction was repeated several times with C^{14}-CuCN to give (C^{14}-CN) TCIN with activity of 6065-6300 dpm/ug from C^{14}-KCN of activity 57.81 mCi/mmol, which had been diluted with KCN to 1.18%. This final product activitiy was 107-112% of expected. The mass spectrum of C^{14} -TCIN is shown in Fig. 5.12, and is identical to that of TCIN.

It should be noted in this context that carbon-14 labelling of CN functions can be carried out by a combination of thermal decomposition and neutron irradiation of Cu_3N (Stoecklin and Vogelbruch, 1968). C^{14}-CN benzonitrile has been prepared by this route (with $\approx$ 10% radiochemical yield), although further substitution has not been attempted.

In conclusion, the use of Route 4, with iodine as the leaving halogen, with an overall radiolabel yield of 65% from a relatively cheap starting material, is recommended for the synthesis of (C^{14}-CN) TCIN. It was used to synthesize C^{14}-TCIN for radiotracer studies on the compound's metabolism in fish.

CHAPTER 6

UPTAKE, ORGAN DISTRIBUTION AND EXCRETION OF TCIN BY SALMO GAIRDNERI

6.1.1 INTRODUCTION

By examining the distribution of pesticide residues in different organs, some idea may be gained of modes of detoxication, sites of accumulation and toxic action of the pesticide. Use of radiolabelled material allows study of the pharmacodynamics of the radiolabelled moiety even when transformed to bound and metabolized forms of the parent compound. As a consequence this gives much more information than a study based only on the parent compound. It also allows greater sensitivity since much lower concentrations of residues can be assayed than with G.C. analysis. This is particularly important in this study where exposure concentrations are low (0 - 20 ug/l).

Previously published work on the biological fate of TCIN includes studies of its fungitoxic action (Turner, 1969; Tillman et al., 1973), its ability to conjugate with glutathione in vitro and in fungal cells (Vincent and Sisler, 1968; Tillman et al., 1973), its hydrolysis to phenolic and amide derivatives (Szalkowski and Stallard, 1977), its interactions with thiol-rich proteins (Long and Siegel, 1975), and its interactions with rat DNA and histones (Rosanoff and Siegel, 1981). Studies of its toxicology in higher organisms have been limited to lethal level and basic metabolism studies in mammals (Chin et al., 1981; Gutenmann and Lisk, 1967; F.A.O., 1975), and lethal level determinations in several fish species (Nishiuchi, 1977, 1979; Perevoznikov, 1977), as described earlier.

It was decided to study the uptake and elimination of C^{14}-TCIN in Salmo gairdneri during continuous-flow exposure at a sublethal level of 10 ug/l.

6.1.2 MATERIALS AND METHODS

Experimental procedures

C^{14}-CN labelled TCIN was synthesized by the method reported in Chapter 5. Radiochemical purity was established by TLC and G.C. at 98%. Specific activity was 6300 dpm/ug (0.734 mCi/mM). Stock solutions were made in acetone.

Salmo gairdneri (5-8 g) were acclimated for ten days in the flow-through system described earlier at 9 - 11° C, with daily feeding to satiation with pellet feed. Feeding was stopped 24 h prior to exposure. During an exposure experiment, radiolabel water stock was pumped in with the inflowing water supply, which was set at a rate sufficient to maintain oxygen levels at $\geqslant$ 70% saturation. Water samples were taken twice daily for extraction and radioanalysis during exposure periods. Aquarium C^{14}-TCIN concentrations were maintained at 10 ug/l.

Two groups of fish were exposed to C^{14}-TCIN over 96 h. During exposure, four fish were taken for analysis at each of 6, 12, 24, 48 and 96 h. At 96 h, the second group of fish were washed and transferred to 20 l aerated uncontaminated water. Four of these fish were sampled at each of 6, 12, 24, 48 and 96 h of depuration. Water was changed at 24 h. Water samples were taken at intervals during the depuration phase.

This experiment was performed twice. Depuration rates were also measured for another group of eight S. gairdneri over 96 h when placed in 20 l aerated, fresh water, after exposure to 10 ug/l C^{14}-TCIN solution for 96h.

Analysis of fish samples

Fish were anaesthetized (tricaine methanosulphonate), severed caudally, and blood was collected. Organs were dissected out, weighed, solubilized (Soluene $^{(R)}$), and radiocounted in 5 ml Dimilume-30 (Packard) on a Packard Prias-PL Tricarb scintillation counter. Counts were quench corrected on the basis of a quench curve established with C^{14}-toluene standards in Dimilume-30 with a range of volumes of carbon tetrachloride added. Organs analyzed were: spleen, kidney, liver, gall bladder, white muscle, skin, hind-gut, and fat and caecal tissue. Blood was centrifuged in haematocrit tubes. Plasma

and whole blood were radioassayed.

In the first exposure experiment, organs were pooled for each sample. All liver samples were divided in two. One half was radioanalysed whole. The other was homogenized in 5 ml distilled water, 0.5 ml 10% trichloroacetic acid were added and after standing overnight at 4° C, the mixture was centrifuged at 2000 g for 30 min to give a clear supernatant and a protein pellet. The pellet was washed in acetone, recentrifuged, solubilized and radiocounted.

In the second experiment, all organs were analyzed individually. At 12 and 96 h exposure, whole body analyses were also performed.

Analysis of water samples

For the uptake phase, 240 ml water samples were extracted with 10 ml redistilled hexane by magnetic stirring for 20 min (extraction efficiency, 96% by internal standards). A 2 ml aliquot of the hexane fraction was radiocounted as above

Water from the depuration phase was extracted as above. Both hexane and aqueous phase aliquots (2 ml and 1 ml respectively) were radiocounted.

6.1.3 RESULTS

Uptake phase

All fish were found to take up TCIN to significant levels at 6 h and onward. Levels of TCIN increased with time in all organs, although responses differed (Figs. 6.1 and 6.2).

In exposure experiment 1, gall bladder levels rose rapidly with time to a 96 h level of 4,400 ug/g, a concentration factor of 4.4×10^5. Hind gut levels were next highest, rising steadily with time, probably caused by bile release. Gill and liver levels also rose rapidly. Levels in the liver were enhanced by protein binding. Protein-bound C^{14}-TCIN comprised approximately 50% of total label in the liver at 96 h (Fig. 6.3). Accumulation in fat and caecal tissue was of the same order as in the liver.

Levels were lowest in muscle, spleen, blood and skin. 96 h

concentration factors are shown in Table 6.1, based on label equivalents of C^{14}-TCIN. Apart from muscle and spleen, concentration factors were all greater than 1000.

The results of exposure experiment 2 confirm those in experiment 1. Individual sample counting eliminates sample-size bias, and consequently allows a better estimate of concentration trends. Gall bladder levels were again highest, rising to a peak at 24 h post exposure of 665 ug/g. At 96 h exposure, the level had fallen to 257 ug/g, possibly due to bile release. This would have caused a decrease in whole gall bladder count without being necessarily related to a decrease in bile count. All gall bladders were full during both experiments except at 96 h in exposure 2 where three out of four fish showed signs of biliary depletion.

Gill levels of TCIN are recorded as ug/g whole gill, including gill arch. Since gill arch is largely supportive material, TCIN accumulation is likely to occur by association with the metabolically active tissue of the filaments. When corrected for gill filament weight only, gill TCIN levels are higher by a factor of 1.83, and are thus comparable with levels occurring in the liver. TCIN accumulation in the filament tissue may, therefore, have a significant effect on gill functioning, especially if the association is due to protein binding or accumulation of toxic metabolites.

Percentile distribution of TCIN in fish exposed for 12 and 96 h are shown in Table 6.2. Gall bladders, despite the low organ weight, are the main TCIN compartment within the body, and maintain a content of 66 - 68% with increasing time of exposure. The hind gut increases by a factor of 10 in importance as a TCIN compartment from 12 to 96 h. All other organs decrease in relative TCIN content with time, although their TCIN concentrations increase.

Depuration phase

Results for exposures 1 and 2 are also shown in Figs. 6.1 and 6.2, and have similar trends. Gall bladder levels dropped markedly after placing fish in clean water, but were still high at 96 h. Hind gut levels rose to a peak at 24 h post exposure and then fell. This is probably a consequence of biliary release and hepatic recycling. Biliary levels peaked 24 h earlier in exposure 1 than in exposure 2. Levels in gills dropped rapidly in exposure 2 on depuration, whereas

they appeared to reach a plateau of 21 ug/g whole gill in exposure 1. The levels for both experiments were of the same order.

After transfer to clean water, blood levels dropped exponentially. Skin and fat levels also dropped, maintaining moderate levels at 96 h depuration. Muscle levels remained around 1 ug/g. Liver and kidney levels appeared to increase initially after transfer, but dropped after 24 h in clean water. Spleen levels increased linearly with time during exposure and increased at the same rate during depuration.

Liver protein binding of C^{14}-TCIN was considerable, comprising some 50% of bound level at all times. Values peaked early in exposure and appeared to attain a plateau. An increase in bound and cytosolic C^{14}-TCIN was observed at 24 h depuration with a subsequent decrease. Cytosol counts were not made during the mid-exposure period due to suspected contamination with bile on dissection.

Excretion

Water analysis gave measures of the aqueous and hexane soluble fractions, equivalent to the total polar metabolites and parent compound levels respectively. The time-dependent aqueous count rates were converted to ug/100g fish of C^{14}-TCIN equivalents, and are plotted against time for both exposures in Fig. 6.4. Excretion was constant with time over the 96 h depuration period, and showed no sign of decreasing. The excretion rate of fish from exposure 1 was higher than those from exposure 2. This may be related to a lower body load reflected by lower gall bladder levels of C^{14}-TCIN in exposure 2 at 96 h.

The excretion plots are based on counts of the water soluble metabolite fractions from the excretion tank water. The levels of TCIN based on hexane counts were < 0.01 ug/l ($< 0.5\%$ total count) at all times, and did not increase with time. Thus, there is no effective excretion of the parent TCIN.

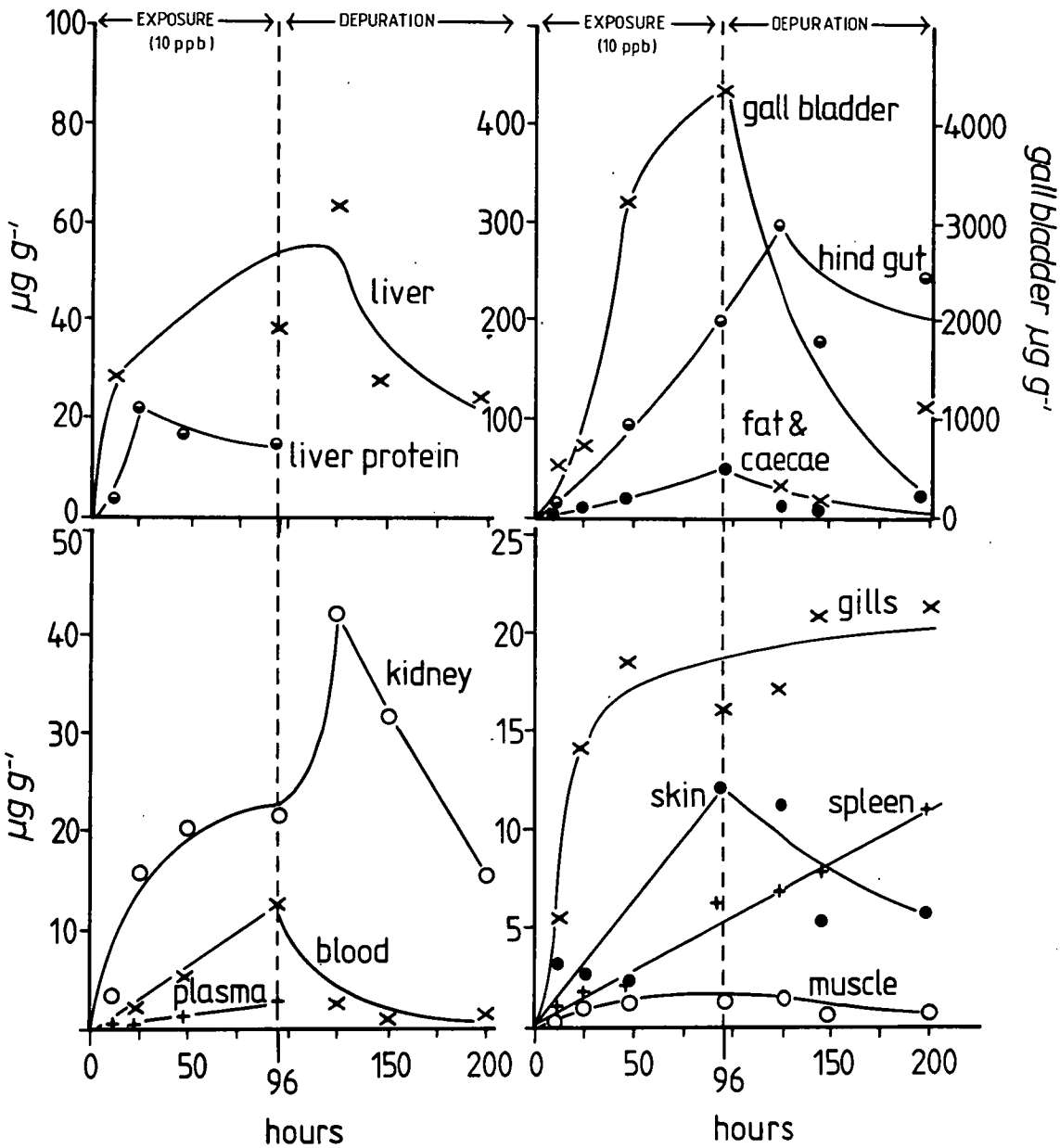


Fig. 6.1 Exposure experiment 1: Levels of C^{14}-TCIN in various organs of S. gairdneri during exposure to 10 ug/l C^{14}-TCIN, and subsequent depuration in fresh water.

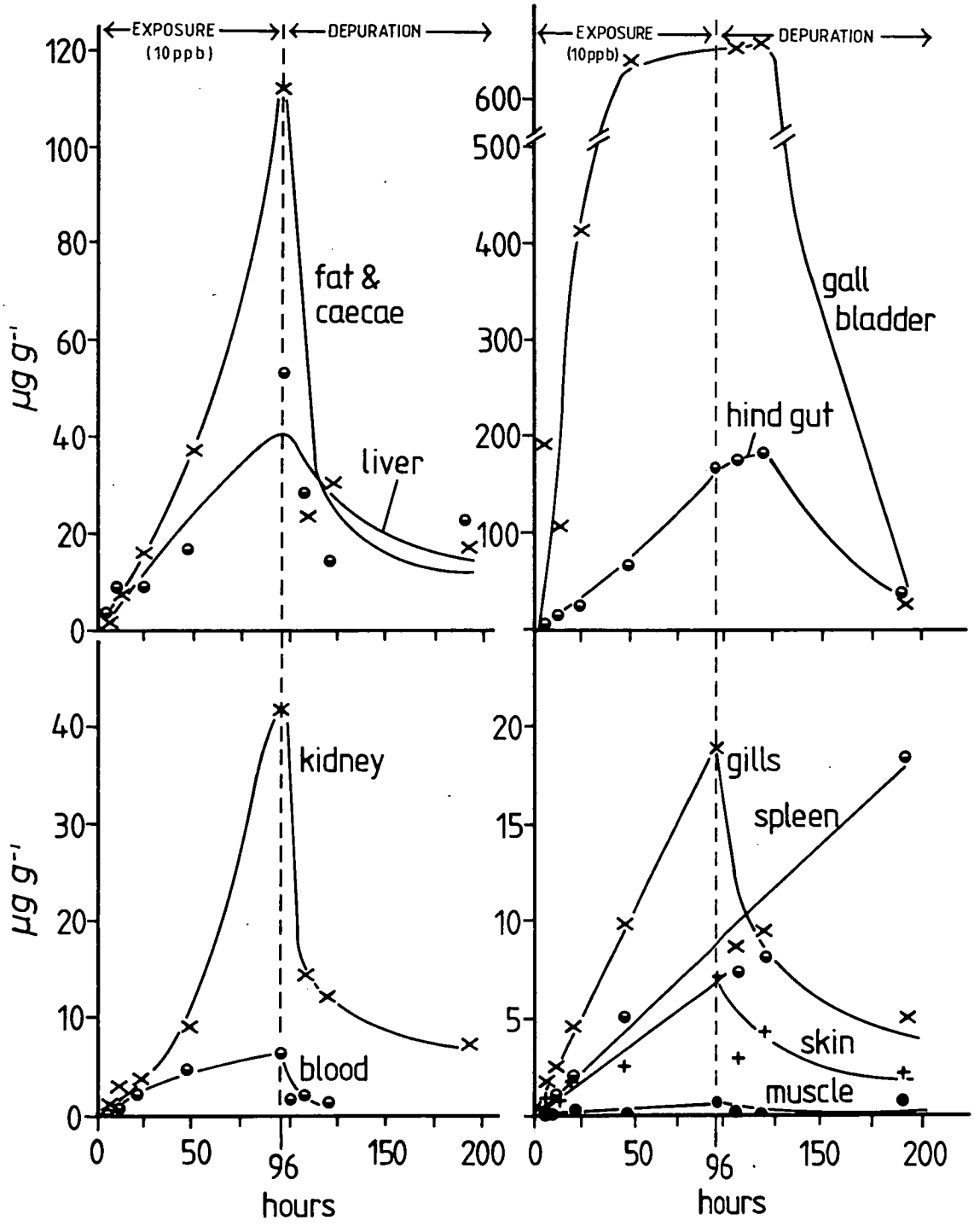


Fig. 6.2 Exposure experiment 2: Levels of C^{14}-TCIN in organs of S. gairdneri.

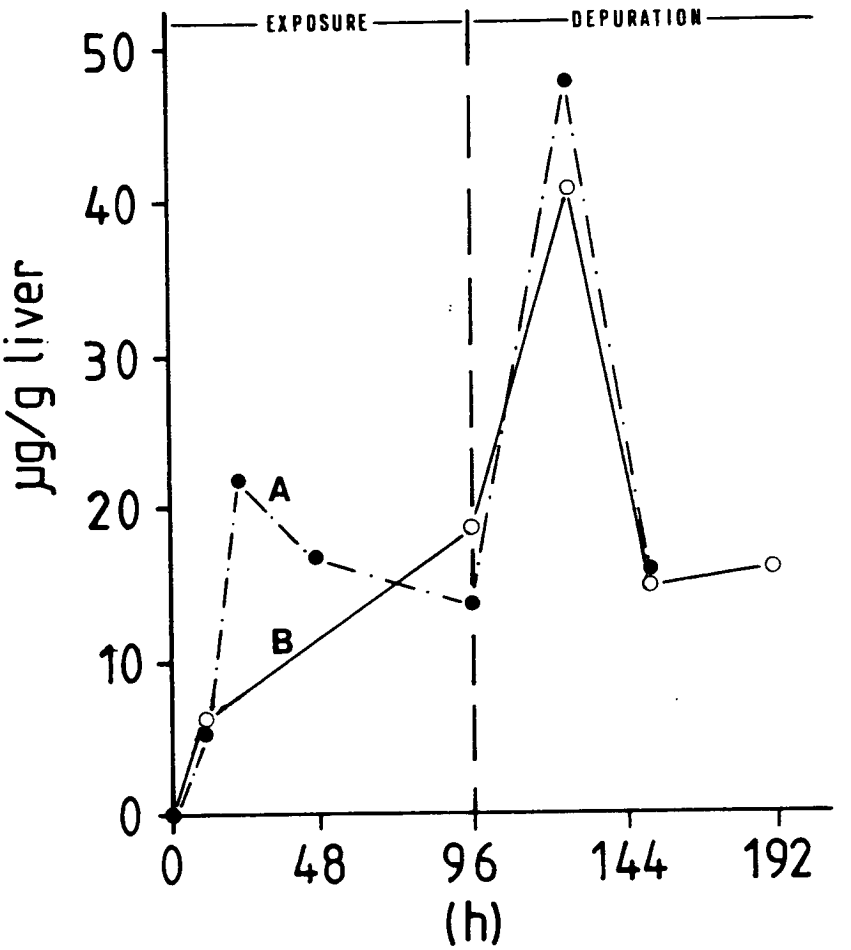


Fig. 6.3 Distribution of TCIN in the liver of S. gairdneri during 96 h exposure to 10 ug/l TCIN and 96 h depuration in clean water.

A = TCIN equivalents in the protein fraction.

B = TCIN equivalents in the cytosol fraction.

Table 6.1 96 h organ TCIN concentration factors in S. gairdneri

Organ	Concentration factors (x 10^3) exposure 1	exposure 2 (SE)
Gall bladder	436.69	25.70 (6.78)
Hind-gut	19.75	16.54 (1.04)
Liver - whole	3.83	5.33 (1.20)
cytosol	1.95	
protein	1.41	
Fat - only		11.11 (2.95)
plus caecae	4.93	
Kidney	2.24	4.02 (1.05)
Gills	1.60	1.91 (0.52)
Spleen	0.69	1.65 (0.75)
Muscle	0.94	0.74 (0.12)
Skin	1.20	0.70 (0.23)
Blood - whole	1.24	0.61 (--)
plasma	0.18	

Table 6.2 Distribution of TCIN in S. gairdneri exposed to 10 ug/l TCIN for 12 and 96 h (%).

Organ	12 h	96 h
Gall bladder	66.0	68.0
Hind-gut	1.3	11.0
Liver	3.1	1.2
Fat and caecae	0.5	1.7
Gills	2.6	0.9
Remainder	23.5	15.0
Mean body burden (ug/g)	2.66	19.15

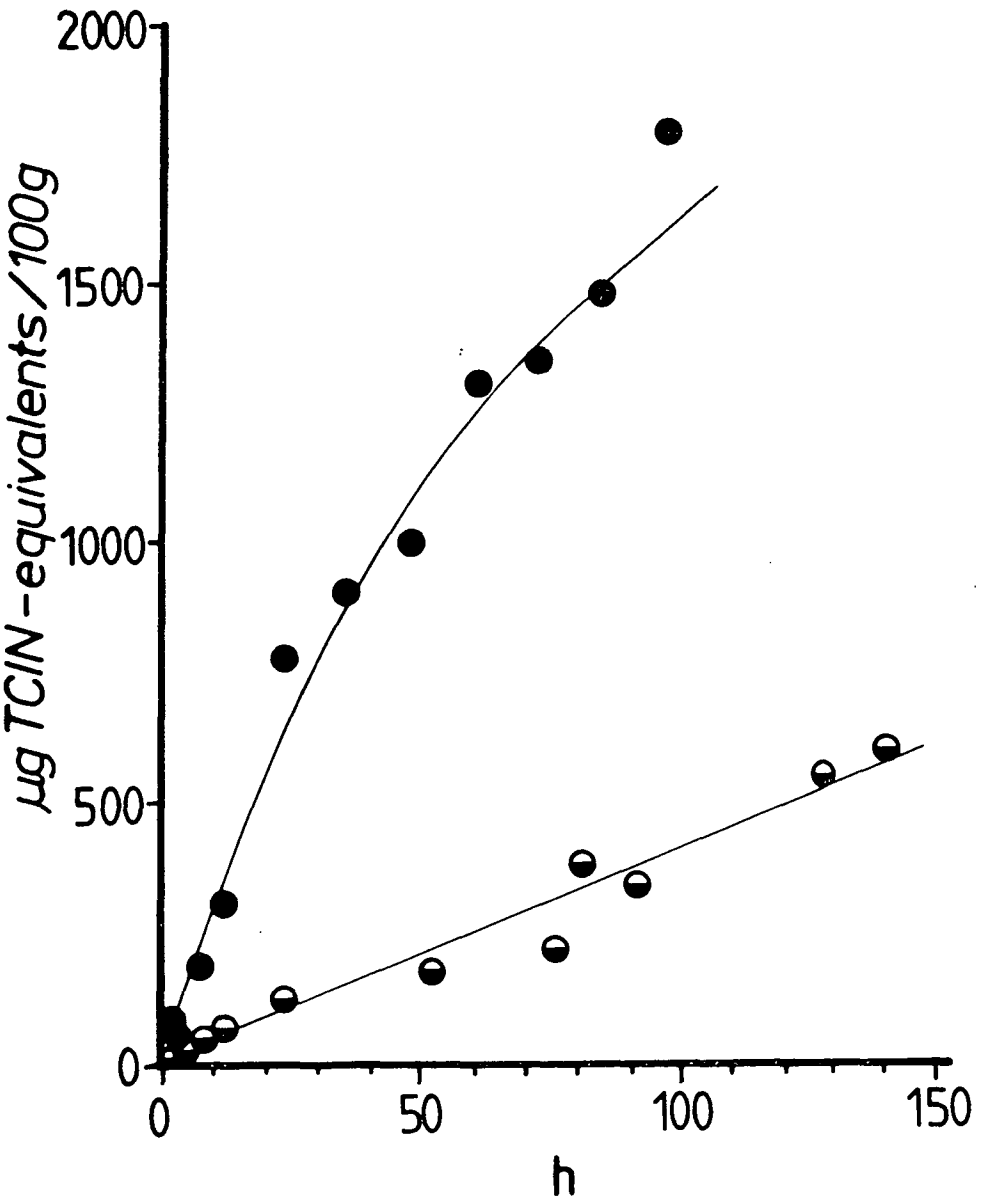


Fig. 6.4 Excretion of C^{14}-TCIN polar metabolites by S. gairdneri in fresh water, previously exposed to 10 ug/l C^{14}-TCIN for 96 h.

● = Exposure experiment 1.

◒ = Exposure experiment 2.

6.1.4 DISCUSSION

Glutathione conjugation and thiol-binding ability are already well established for TCIN in vitro and in fungi (Vincent and Sisler, 1968; Tillmann et al., 1973; Long and Siegel, 1975). The occurrence of high gall bladder levels of radiolabel material in C^{14}-TCIN exposed S. gairdneri was in accordance with this behaviour, since the bile is regarded as the main site for release of glutathione conjugates and derivatives (Arias et al., 1982). Recycling via the digestive system would appear probable, and may explain the constant release rate of polar metabolites during depuration. Glutathione conjugation may be the first step in the production of mercapturic acid conjugates of TCIN, released after a combination of hepatic recycling and renal metabolism (Boyland and Booth, 1962). The gall bladder is a well established site for excretion of polar conjugates of xenobiotics in fish (Kobayashi, 1979; Statham et al., 1976).

Significant binding of TCIN by hepatic proteins occurs. It may also occur in the gills, and could be related to respiratory stress observed in acutely exposed fish and to the gill damage caused by TCIN on prolonged low level exposure. Hepatic protein binding in S. gairdneri may be to thiol-rich proteins such as glyceraldehyde 3-phosphate dehydrogenase, whose binding and inhibition by TCIN have been reported by Long and Siegel (1975). The toxic action of TCIN may be due to enzyme inhibition or glutathione (GSH) depletion followed by reactions with thiol-rich proteins. The observation that significant protein binding occurs within 6 h of exposure appears to indicate that such binding is independent of the GSH pool. The final (96 h) non-protein liver radiolabel counts give a total level of 0.1 - 0.2 mM TCIN, capable of binding 0.1 - 0.4 mM GSH. Glutathione levels in fish liver are commonly 0.5 - 2 mM (Chapter 8; Bauermeister et al., 1983). Total levels of 2 - 10 mM TCIN metabolites were observed in the bile, indicating a major flux of GSH from the liver. Depletion of GSH levels depends on the availability of bound GSH and GSSG, the rate of GSH synthesis and, consequently, the nutritional state of the animal (Arias et al., 1982). The latter would be directly related to exposure since acutely TCIN-stressed fish do not take food (4.1.3).

It is of interest to compare the results of this work with those of one of the most detailed investigations of organic xenobiotic uptake and metabolism in fish, the work of Kobayashi on phenols in Carassius auratus. Kobayashi (1979) elucidated the metabolic pathways of pentachlorophenol (PCP) and phenol detoxication. They involved β -glucuronidation and sulphate conjugation followed by both renal and gill excretion.

As in the present study, the gall bladder was the principle compartment for both phenols (Kobayashi and Akitake, 1975; Kobayashi et al., 1976). The concentration in the gall bladder increased with time during exposure, and was also observed to increase with time of depuration in PCP exposed fish over the first 24 h in clean water (Fig. 6.5). This was also observed in TCIN exposed fish in exposure 2, followed by a rapid decline, which had commenced at less than 24 h of depuration in exposure 1.

There are important differences between the behaviour of phenol and the less soluble PCP, which can be extended to the behaviour of TCIN. The exposure concentration of PCP in Kobayashi's experiments was 200 ug/l, whereas that of phenol was 15 mg/l. Despite this large difference, the equivalent organ concentrations of both compounds during exposure were roughly equal. Gall bladders of PCP exposed fish were, however, at a much higher concentration than those exposed to phenol at all times. Also, the other organs showed a much lower rate of depuration in the case of PCP than with phenol (Kobayashi and Akitake, 1975; Kobayashi et al., 1976). Kobayashi et al. (1976) suggested that such differences are due to the higher solubility of phenol affecting drug elimination.

The dynamics of TCIN are much more in accord with those of PCP than with those of phenol. Gall bladder levels were 412 and 750 ug/g at 24 h for the two TCIN exposure experiments, of the same order as for PCP, despite the TCIN exposure concentrations being twenty times lower. Other organ TCIN levels were also of the same order as for PCP exposed fish in Kobayashi's work, although PCP levels appeared to plateau at much shorter exposure times. Depuration of TCIN was slower than for PCP, which in turn demonstrated slower depuration than phenol.

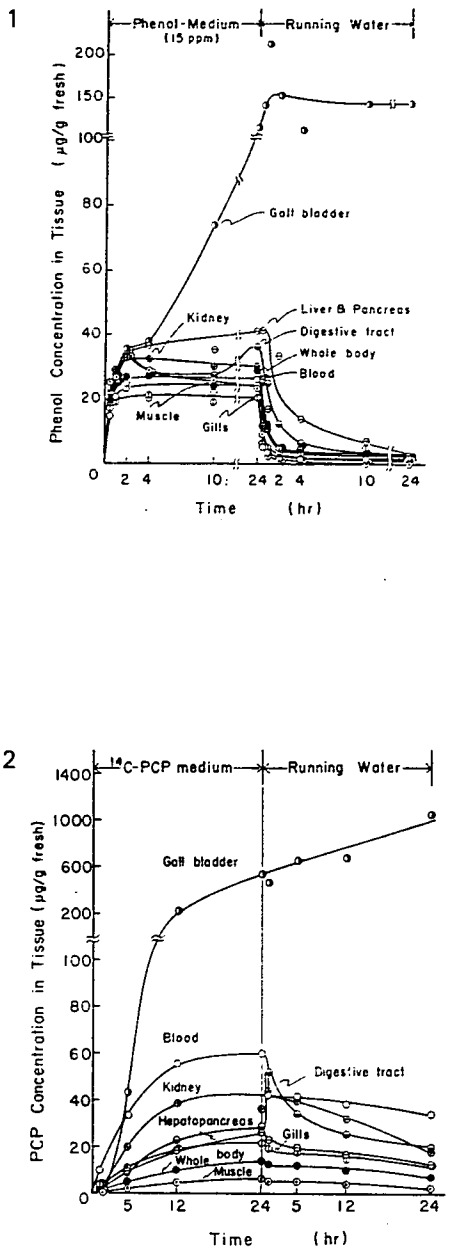


Fig. 6.5 Organ levels of PCP (2) and phenol (1) in Carassius auratus during exposure and depuration. (From Kobayashi and Akitake, 1975 and Kobayashi et al., 1976).

It appears, therefore, that the pharmacodynamics of pesticides in fish are profoundly influenced by their solubility, as is bioconcentration (Chiou, 1981). Higher organ concentrations and slower depuration rates probably reflect the affinity of the low water solubility, high lipophilic, chlorinated pesticides for organic material and fats. Tooby et al. (1974) quoted higher levels of muscle and lower levels of liver accumulation of dichlobenil (2,6-dichlorobenzonitrile), a less chlorinated and more soluble analogue of TCIN, over six days of exposure to 1 mg/l in S. gairdneri. Depuration rates were more rapid than for TCIN.

The accumulation of pesticides in various organs must also be influenced by the rate and mode of detoxication reactions. The rate of detoxication of TCIN in S. gairdneri must be relatively high since gall bladder residue levels drop rapidly after 24 h depuration. The rate of accumulation of residues in the bile implicates an enzymatic metabolic step, since the lipophilic parent compound is unlikely to be accumulated in the highly polar bile without being in a polar form (Arias et al., 1982), and is unlikely to react with nucleophiles at a rate sufficient to equal that observed for biliary accumulation in S. gairdneri, without some form of catalysis.

TCIN could be metabolized and converted to polar conjugates by reaction with either thiol or hydroxyl nucleophiles. The rate of reaction of TCIN with glutathione and hydroxyl groups is low at physiological pH (Vincent and Sisler, 1968; Szalkowski and Stallard, 1976). Three groups of enzymes could be invoked in TCIN metabolism, the glutathione S-transferases, the β-glucuronidases and the sulpho-transferases, all of which are considered as Phase II detoxication enzymes (Chambers and Yarbrough, 1976). However, positive identification of the biliary metabolites of TCIN must be made before an understanding of the detoxication processes can be reached. This is the subject of Chapter 7.

CHAPTER 7

BILIARY METABOLITES AND PROTEIN BINDING OF TCIN

7.1 IDENTIFICATION OF TCIN METABOLITES

7.1.1 INTRODUCTION

As outlined in Chapter 1, there are a large number of metabolic processes involved in the detoxication or toxic activation of organic xenobiotics in fish, few of which have been elucidated in any depth. Two processes have, however, been examined in some detail. These are the monooxygenase metabolism of the cytochrome P450 system, and the glucuronidase conjugation system (Chambers and Yarborough, 1976). No conclusive evidence for the existence of mercapturate pathways in fish has so far been presented. In a recently published paper, Bauermeister et al. (1983) demonstrated the existence of glutathione S-transferase and γ-glutamyl transpeptidase activity in various organs of Salmo gairdneri, and suggested the existence of mercapturate metabolism in this species. The process of conversion of glutathione conjugates to cysteinyl adducts with consequent acetylation to the mercapturic acids (Fig. 7.1) is well known in mammals as a major phase II detoxication pathway (La Du et al., 1972). The only secondary evidence for this pathway in fish is the generation of cysteinyl and mercapturate metabolites of a range of hydrocarbons in marine fish and squalids (Bend et al., 1979), the identification of the glutathione conjugate of acetaminophen on incubation with isolated hepatocytes of S. gairdneri and its degradation by kidney homogenate (Parker et al., 1980, 1981), and the identification of the mercapturate of molinate in fish bile (Lay and Menn, 1981).

The fungitoxic action of TCIN has been attributed to its ability to conjugate with and deplete levels of glutathione in fungal cells (Tillman et al., 1973). Two main routes for detoxication of TCIN, therefore, suggest themselves (Fig. 7.2). TCIN may be converted to the glutathione conjugate(s) with secondary conversion to cysteinyl and mercapturate conjugates, or it may be converted to the 4-hydroxyl

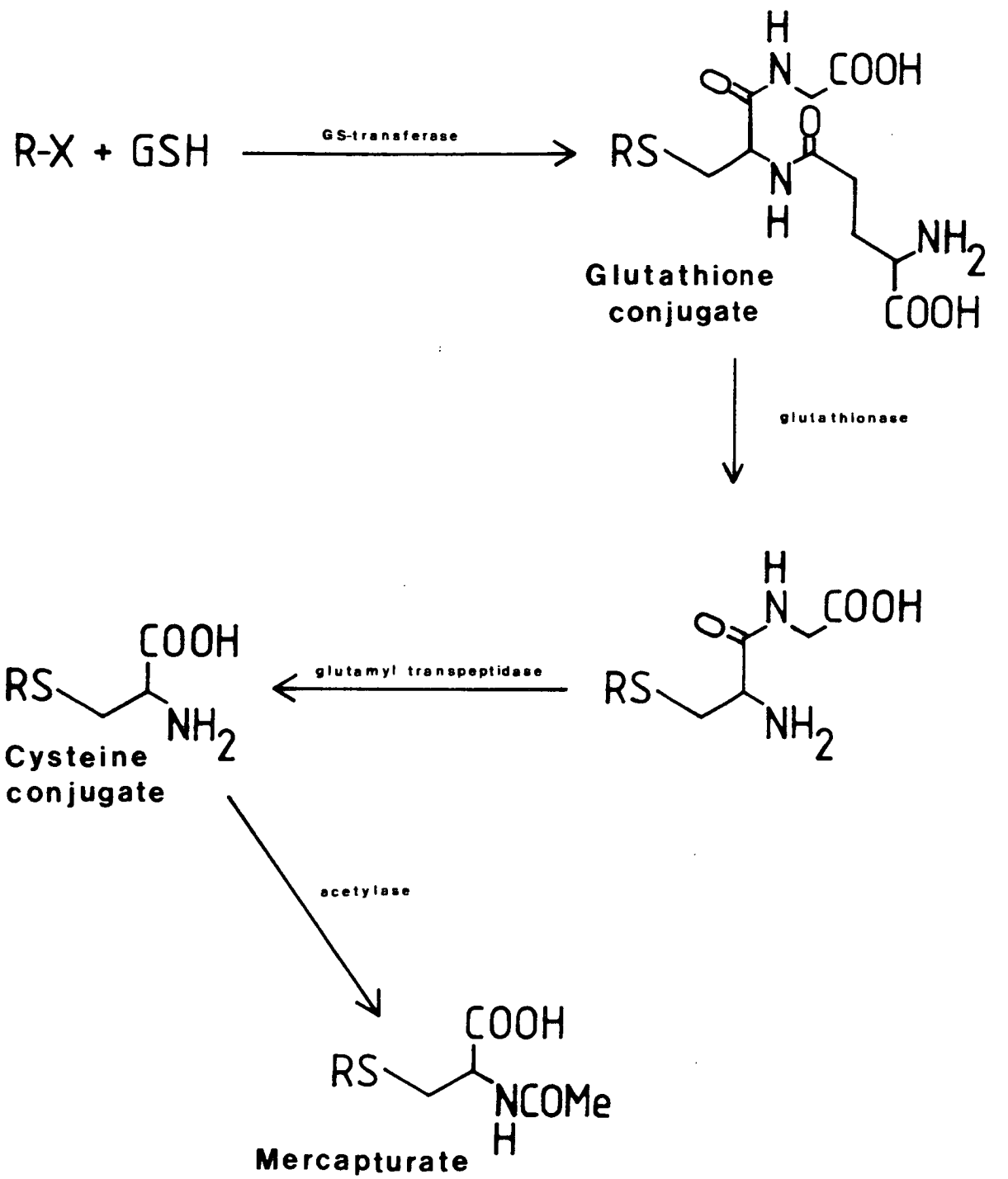


Fig. 7.1 Mercapturate pathway of drug metabolism.

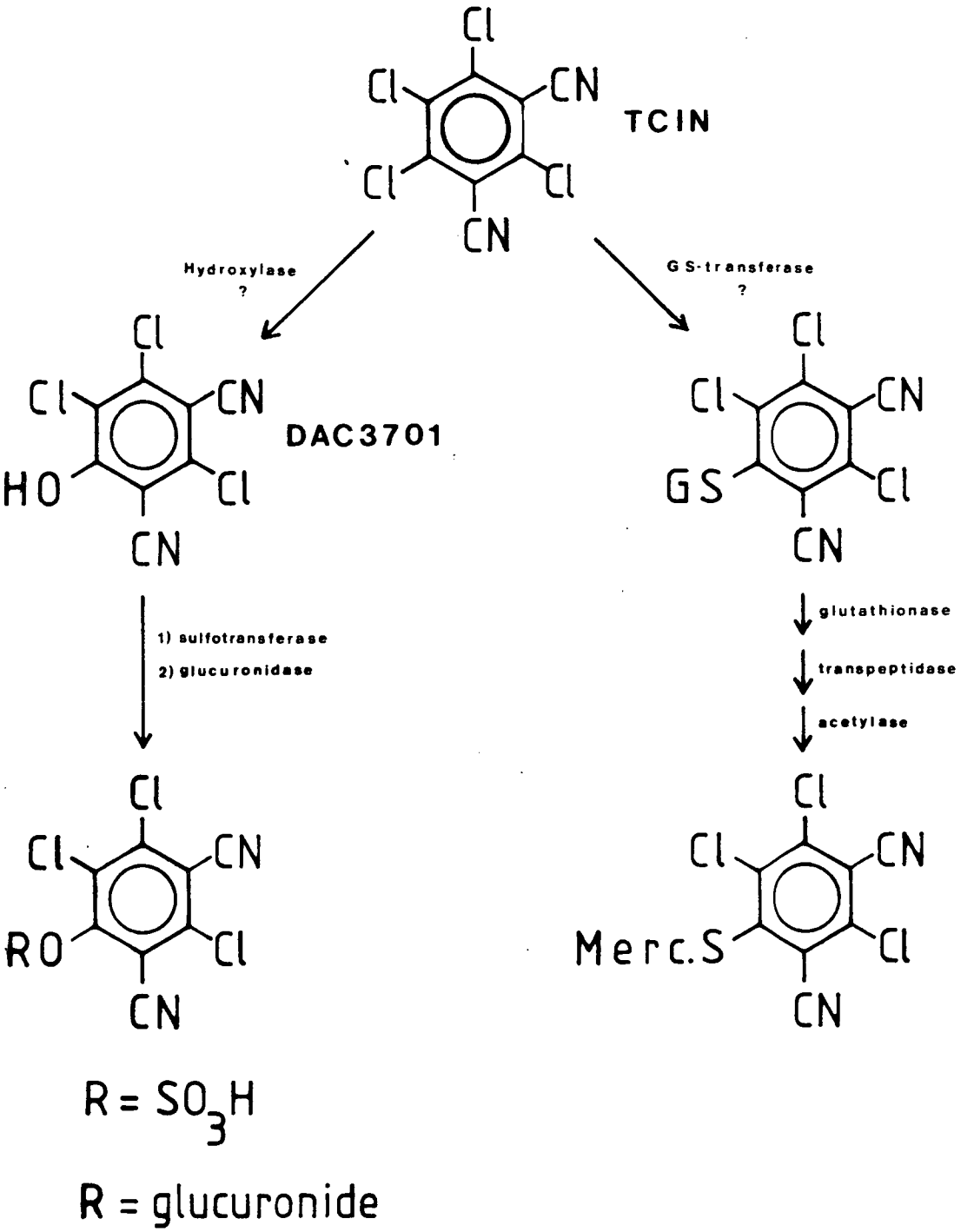


Fig. 7.2 Possible routes of TCIN metabolism.

phenolic derivative, DAC3701, and subsequently conjugated with β -glucuronide and/or sulphate, as occurs in the detoxication of PCP (Kobayashi, 1979). The observation of accumulation of TCIN in bile and excretion of polar metabolites in exposed fish (Chapter 6) are in accordance with both of these metabolic routes.

In order to investigate the type of TCIN metabolites produced by TCIN-exposed Salmo gairdneri, bile was examined by a combination of thin layer chromatography and gel-column chromatography.

7.1.2 MATERIALS AND METHODS

Chemicals and apparatus.

All chemicals were A.R. grade unless otherwise specified. Solvents were A.R. grade or redistilled. Dimilume-30 and Soluene were obtained from Packard Pty. Ltd.. Mass spectra were obtained on a Micromass 7070F double focussing spectrometer. Fourier Transform Infrared spectra were obtained on a Digilab FTIR spectrometer. N.M.R.(H) spectra were obtained at 100 MHz on a Jeol JNM-4H-100 spectrometer, tetramethylsilane as internal standard. U.V. and visible absorbance measurements were made on a Pye Unicam SP6-550 UV/VIS spectrometer.

Exposure and sample collection

Salmo gairdneri (mean weight 9.0 g) were provided by Sevrup Fisheries Pty. Ltd., Bridport, Tasmania. Acclimation and exposure procedures have already been described elsewhere (3.1.2). Bile and livers were collected from trout exposed to 10 ug/l C^{14}-TCIN (activity 6300 dpm/ug) for 96 h, snap frozen in liquid nitrogen, and stored at -18° C. Liver cytosol preparations were made by homogenizing livers in 8 vol. 0.1 M Tris-HCl buffer (pH 8.3) and centrifuging at 30,000 g for 2 h.

Metabolite identification

Bile was subjected to thin layer chromatography (TLC) at time periods between 0 and 120 min of standing at 20° C. Two TLC systems were used:

System 1: Whatman No.2 chromatography paper; (descending); n-propanol-acetic acid-water, 9:1:3.; developed to 35 cm.

System 2: Merck Kieselgel 60 F_{254} plates, (ascending); benzene-acetone-methanol, 2:1:5; developed to 19 cm.

Amino acid spots were visualized by spraying with Ninhydrin reagent and heating. All TLC runs were cospotted with a mixture of TCIN-glutathione conjugates, freshly prepared from TCIN and excess (6x) glutathione in aqueous methanol, 5% in K_2CO_3. TLC standard mixtures were also prepared from C^{14}-TCIN and glutathione in the same way.

Both paper and silica chromatograms were divided into 5 mm strips and cut or scraped into scintillation vials, extracted with aqueous methanol (1:1) for 2 h with shaking and radiocounted on a Packard Prias-PL Tricarb scintillation counter in 5 ml Dimilume-30.

Gel chromotagraphy

Liver cytosol preparations and bile were subjected to gel column chromatography on Sephadex G75 at 1 ml/min on a 2.6x30 cm column equilibrated and run with 0.1 M Tris-HCl buffer (pH 8.3). Samples of 4 ml each were collected and the absorbance of each fraction was measured at 240 and 280 nm. Aliquots of fractions were radioanalysed in Dimilume-30. Protein analyses were performed using the Coomassie Brilliant Blue method of Spector (1978) with bovine albumen as the standard. The gel column was calibrated using GmBH Combithek standard proteins.

The low molecular weight fractions of the bile run were combined, freeze-dried and analysed on TLC system (1).

4-hydroxy trichloroisophthalonitrile, DAC3701, was synthesized from TCIN by the method of Heilman et al. (1978). The sulphate ester potassium salt of DAC3701 was synthesized from DAC3701 by the general method of Feigenbaum and Neuberg (1941). On aqueous recrystallization, an 85% yield of colourless cubic crystals was obtained. I.R. 1100 cm^{-1}br., (SO_3^-); 2080,2150 cm^{-1}(Ar CN str.).

These two compunds were used as TLC standards. A mixture of cysteine-TCIN conjugates was also prepared as for the glutathione-TCIN conjugate mixtures, for TLC standards.

7.1.3 RESULTS

The Rf values of glutathione-TCIN conjugates, cysteine-TCIN conjugates, DAC3701 and its sulphate ester derivative are shown in Table 7.1 along with those of the radioactive peaks in the bile chromatograms. The chromatogram (System 1) of freshly prepared bile (Fig. 7.3, 1) shows two main peaks corresponding to the mono- and di-glutathione conjugates, G1 and G2 respectively, which comprise 65% of the total biliary radiolabel. The remaining 35%, at higher Rf, is unidentified. By silica gel TLC (System 2), it was found that biliary metabolites consisted of only 0.79% of TCIN and 0.36% of the phenolic DAC3701. No activity in chromatograms of freshly prepared bile could be attributed to the sulphate ester of DAC3701 by TLC evidence alone.

On standing, the bile radio-metabolite complement becomes more complex with the formation of at least two secondary metabolites. A and B (Fig. 7.3, 2), which were tentatively identified as the cysteinyl conjugates. Fig. 7.3, 3 shows bile after standing 2 h with internal standards added. The high level of radioactivity near the origin may be due to a multiple combination of tri-conjugates of glutathione and cysteine.

Complex TLC patterns were obtained from bile if left standing for periods greater than 15-30 min after collection from the fish. This phenomenon was almost eliminated if bile was immediately passed through a gel column prior to storage and TLC analysis, and clean chromatograms were often obtained with the general appearance of Fig. 7.3, 1. The mono-glutathione adduct comprised 30-37% of the total radiolabelled metabolites in bile.

Table 7.1 TLC Rf values for TCIN, derivatives, and bile metabolites

Compound	Rf values	
	System 1	System 2
Reference compounds:		
TCIN	0.9	0.9
DAC3701	0.81	0.79
Sulphate ester	0.05 - 0.10	0.0 - 0.05
G 1	0.30	0.67
G 2	0.14	0.10
C 1	0.40	0.70
GSH	0.20	0.39
Bile components:		
"G 1"	0.30	0.65
"G 2"	0.13	0.08
A (C 1 ?)	0.35	0.68
B	0.17	--

Fig. 7.3 Thin layer chromatograms (System 1) of the bile of S. gairdneri exposed to C^{14}-TCIN.

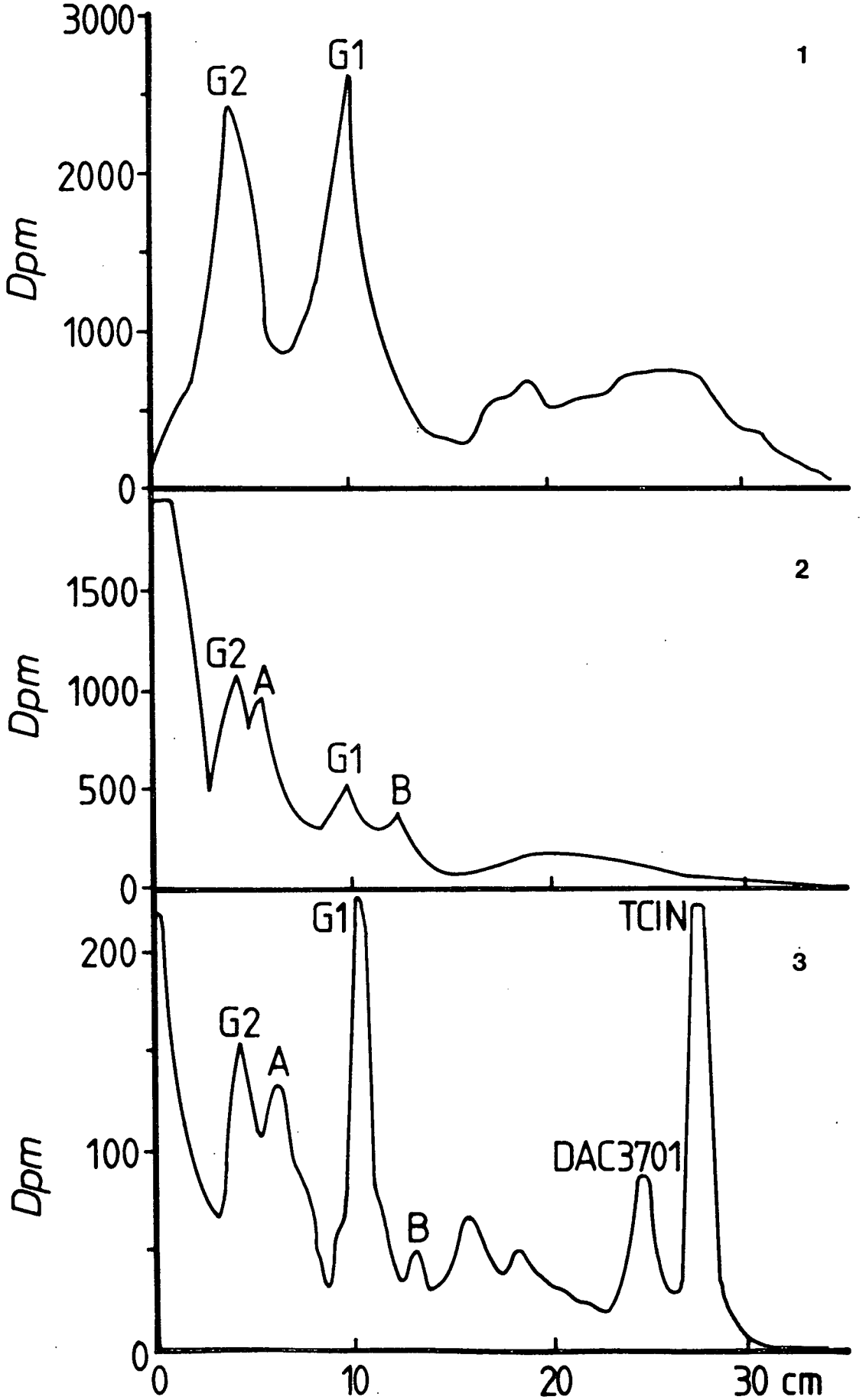
G 1, G 2 = glutathione conjugates of TCIN.

A, B = tentatively assigned as cysteine conjugates.

1 = fresh bile.

2 = bile stood for 120 min at 20° C after collection.

3 = 2 with reference compound mixture added.


3000
2000
1000
0
Dpm
G2
G1
1
1500
1000
500
0
Dpm
G2
A
G1
B
2
200
100
0
Dpm
G1
TCIN
G2
A
B
DAC3701
3
0
10
20
30 cm

7.1.4 DISCUSSION

The TLC evidence derived here confirms that the primary metabolites of TCIN are glutathione adducts, and not phenolic derivatives. This indicates that at least the preliminary steps in a mercapturate detoxication pathway are confirmed in Salmo gairdneri (Fig. 7.4). Rapid degradation of glutathione conjugates in bile resulting in a complex mixture of compounds has been reported by Hirata and Takahashi (1981) who examined the metabolism of methylmercury in rats. They suggested that biliary enzymes contributed to the mercapturate pathway producing cysteinyl conjugates from glutathione adduct precursors, and that this was the cause of confusion in previous work on the nature of biliary methylmercury excretion. The action of such biliary enzymes on mixtures of multiple TCIN-GSH conjugates would result in a complex mixture indeed. The generation of a complex TLC pattern from bile on standing, and the prevention of this phenomenon by gel chromatographic separation of the biliary protein fraction appears to confirm this scenario in Salmo gairdneri.

There are several reasons why the thiol group could be favoured over the hydroxyl as a nucleophilic agent in the reaction with TCIN. Streitweiser (1962) listed several important properties influencing nucleophilicity, and the requirements of a good nucleophile:

1. low solvation energy of the Lewis base;
2. high strength of bond to a carbon 2 p orbital;
3. small effective size;
4. low electronegativity of the attacking atom, and
5. high polarizability of the attacking atom.

For all the above parameters except 3, RS^- is a better nucleophile than RO^-. A standard nucleophilic constant, n, has been selected as defining the reaction rate of the nucleophile with MeI in methanol compared with the rate of methanol solvolysis at 25° C, such that:

$$n_{MeI} = \log(k_{nucleophile}/k_{MeOH})$$

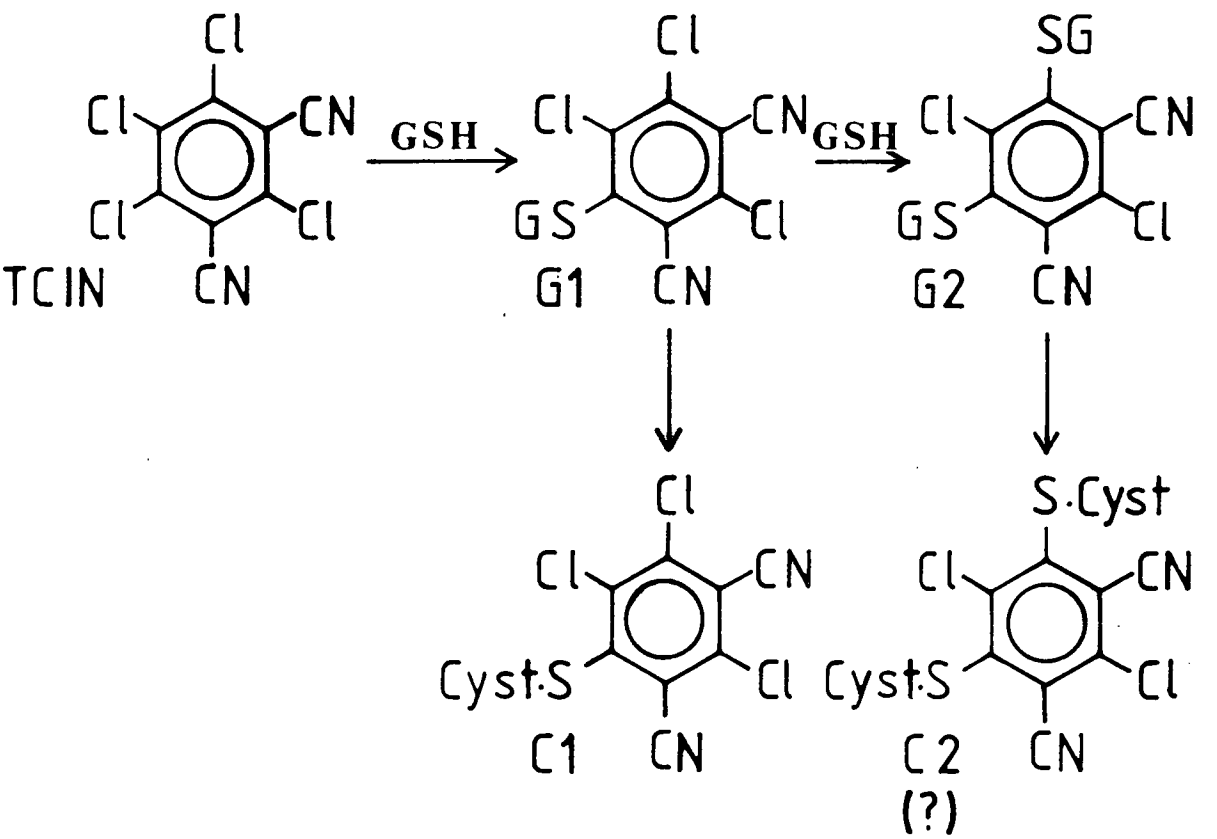


Fig. 7.4 Primary metabolism of TCIN in S. gairdneri.

The following values of n_{MeI} were obtained for a number of nucleophiles (Pearson et al., 1968):

Nucleophile	n_{MeI}
MeOH	0.0
MeO^-	6.3
HO^-	6.5
Me_2	0.25
C_6H_5SH	0.25
$C_6H_5S^-$	9.9

Thiol groups are, therefore, better nucleophiles than hydroxyl groups. This difference is primarily due to the high polarizability of the sulphur orbitals and its lower electronegativity. Biological systems enhance the rate of thiol attack by increasing the effective concentration of thiol groups, and by causing a "solvation specific base catalysis effect", increasing the nucleophilicity of the thiol group by abstracting the proton (Carey and Sundberg, 1978). Such effects have major consequences with regard to the metabolism of strong electrophiles such as TCIN.

Glutathione has been reported to form conjugates with a wide variety of electrophiles including nitroso compounds (Dolle et al., 1980), acrylamide (Dixit et al., 1982), vinyl chloride (Green and Hathway, 1977), acetanilide herbicides (Leavitt and Penner, 1979) and metabolically generated epoxides (Pachecka et al., 1979). It also readily reacts with halogenated aryl compounds (Chasseaud, 1976) to form a conjugate by halogen replacement. Glutathione conjugation processes have been considered to interact with toxicology in three main ways: depletion of glutathione causing toxicosis (Chasseaud, 1976); activation of electrophile to a more toxic form (Rannug et al., 1978); and protection of macromolecules from binding with electrophiles by competition (Buckpitt and Boyd, 1980).

The toxic action of TCIN in fish may be related to its ability to conjugate glutathione in any of the above ways. It is generally considered that electrophile mercapturate metabolites are less toxic than the parent electrophiles due to their higher polarity, and consequent ease

of excretion, and their lessened ability to react further with metabolically important nucleophiles. TCIN is unusual in this respect owing to its ability to form multiple glutathione conjugates. Its electrophilicity may not be significantly reduced on mono-conjugation, and further reaction with other nucleophiles may be significant.

It is useful to compare TCIN metabolites with those observed for compounds of similar structure. Those shown in Fig. 7.5, PCNB, HCB and dichlobenil have been investigated in detail in mammals and cursorily in fish. Renner (1980) found that, in the rat, PCNB was primarily metabolized by glutathione conjugative replacement, not of Cl, but of the nitro function, whose removal is facilitated by the strong electron-withdrawal by chlorines on the benzene ring. This caused many PCNB secondary metabolites to be identical to those produced from HCB, where glutathione conjugation also occurred by replacement of a chlorine. Bahig et al. (1981) showed that the majority of metabolites of PCNB in fish were sulphur metabolites or the methylated thiophenol derivative. It is interesting to note in this respect that pentachlorophenol, pentachloroaniline and pentachlorobenzene do not produce sulfur conjugated metabolites.

The widely used fungicide, HCB, has been found to cause acute porphyria in mammals - a disturbance in heme metabolism leading to a build up of porphyrins in the liver, bile and urine with concomittant haemolytic anaemia. This has been attributed by Koss et al. (1977) to the metabolite pentachlorothiophenol, formed as a secondary metabolite from glutathione conjugated-HCB. It is highly possible that a chlorothiophenolic metabolite may be produced by fish metabolism of TCIN. Despite haemolytic anaemia, TCIN does not appear to cause porphyria in fish. Secondary symptoms of toxic porphyria are loss of porphyrins in the urine giving a red colour, as well as red appearance and red fluorescence under ultraviolet light of the gall bladder, upper gut and occasionally the liver (Granick and Urata, 1963). Investigation showed that no such symptoms occurred. It is possible that the intensification of colour in bile of TCIN exposed fish (4.1.3) is due to a build-up of bilirubin from haemolytic breakdown of blood cells, since the bile is the site of accumulation of bilirubin produced by the liver from free haemoglobin in the blood (Arias et al., 1982). Increases in plasma and biliary bilirubin levels are also

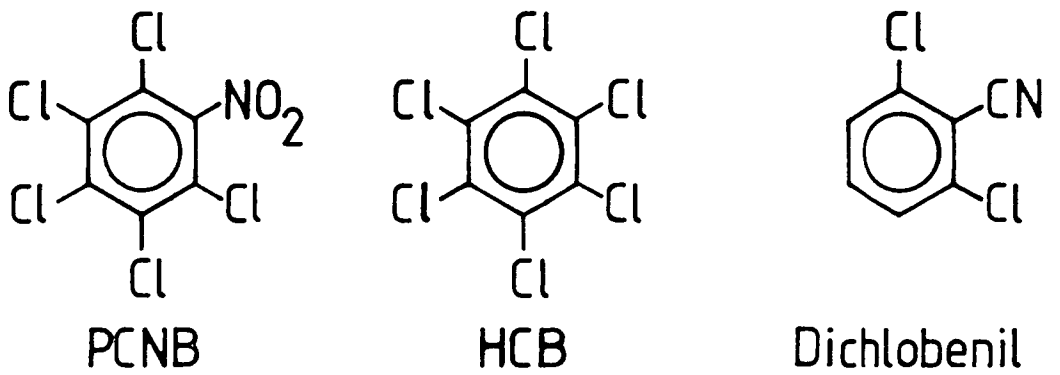


Fig. 7.5 Structures of compounds related to TCIN.

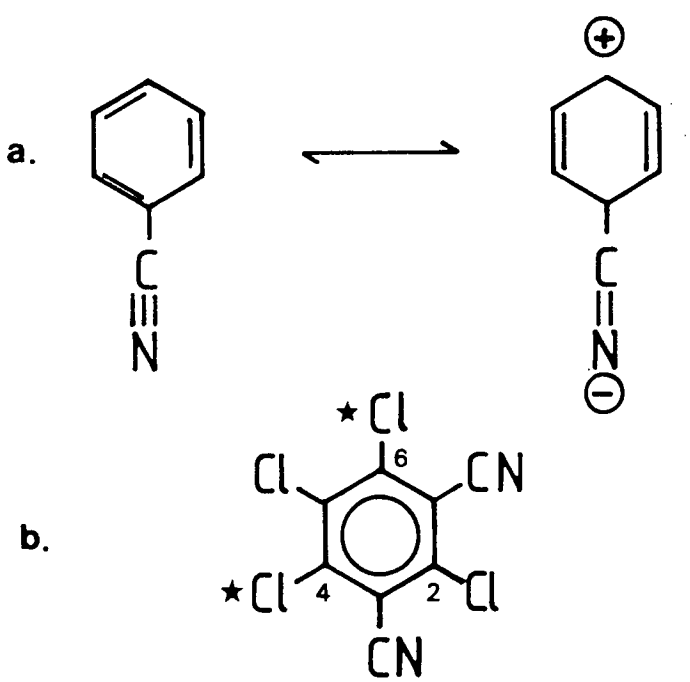


Fig. 7.6 a) Resonance effect of Ar-CN substitution.
b) Sites most susceptible to nucleophilic attack in TCIN (*).

observed on fasting in mammals (Gollan et al., 1975), and hence the similarity in biliary colour changes between TCIN-exposed and fasted fish.

The metabolism of dichlobenil has been investigated cursorily in fish by Verloop et al. (1974), and it is shown to be partially metabolised (5-10%) to the 3-hydroxy phenolic derivative. Since a number of sites on the benzene ring are available for microsomal oxidation, the spectrum of metabolites is expected to be widely different from that for TCIN.

7.2 STRUCTURAL INVESTIGATION WITH MODEL COMPOUNDS

7.2.1 INTRODUCTION

Vincent and Sisler (1968) suggested that glutathione reacted with TCIN by nucleophilic replacement of chlorine in any of three sites, leading to the formation of mono-, di- and tri-conjugation products. The evidence for this reaction sequence was somewhat circumstantial. Infrared spectra of mecraptoethanol thioether analogues confirmed the general nature of the conjugates. No further evidence was put forward, however, to confirm the structure of the glutathione conjugates, and the substitution patterns of the three adducts were not investigated.

The resonance effect of the CN group on the benzene ring causes positions para to them to have lower electron densities (Fig. 7.6) (Carey and Sundberg, 1978). This makes those sites more favorable to nucleophilic attack. This is born out by Hammett plots which give the following δ values for Cl and CN:

	δ_{meta}	δ_{para}
Cl	0.37	0.23
CN	0.56	0.66

where the greater the δ value, the greater the electron withdrawal.

Positions meta to a chlorine atom are more electrophilic than the para position. Consequently, in TCIN, sites 4 and 6 can be regarded as those most susceptible to nucleophilic attack, and this was proposed by Vincent and Sisler (1968). Site 2 is the next most likely site of nucleophilic attack as site 5 is meta to both CN groups and para to site 2 Cl.

A series of thioether analogues of the glutathione conjugates were prepared and further information on the structures of these conjugates was obtained from spectral data for both model compounds and the mono-conjugate with glutathione.

7.2.2 MATERIALS AND METHODS

Synthesis and analysis of model compounds

Model TCIN conjugate analogues were prepared using mercapto-ethanol and ethylmercaptan as model thiols. TCIN, 500 mg, and ethane thiol (2-6 x molar excess) were taken up into methanol, 30 ml. Potassium carbonate, 2-300 mg, was added. The mixture was stirred for 0.5 - 1 h, 3 h and 6 - 12 h, evaporated, triturated with light petrol, extracted with xylene, washed with water, dried (Na_2SO_4) and evaporated to produce thioether mixtures containing predominantly the mono-, di- and tri-conjugate respectively. Mercaptoethanol reaction mixtures were refluxed, and shorter times employed. Preparative TLC was used to separate the conjugates on Merck Kieselgel GF254 in the following solvent systems: benzene-methanol-acetic acid, (90:16:8) for mercaptoethanol, and Lt.petrol(40-60°)-acetone, (9:0.6) for ethanethiol, with multiple development.

Low resolution mass spectra, Fourier Transform Infrared (FTIR) spectra and N.m.r.(H) spectra were obtained for all compounds. Attempts to perform Raney Nickel and sodium in liquid ammonia reductions, by standard methods, were unsuccessful.

The mono-glutathione conjugate of TCIN was prepared essentially as above using methanol-water (1:1) as solvent, refluxing the mixture for 0.5 h. The evaporated residue was subjected to TLC (Kieselgel GF254) with multiple development with methanol-toluene-butanol-water (10:5:5:1), giving a colourless powder. Spectra were obtained as above. Unsuccessful attempts were made to obtain molecular ion peaks

in E.I. and C.I. (NH_3) mass spectra of the pure compound and of the nTFA-nbutyl ester derivative prepared by the method of Roach and Gehrke (1969). FTIR spectra were obtained for glutathione, TCIN, the mono-glutathione conjugate and the above derivative. C^{13} Nmr spectra were determined for the model di- and tri-adducts of ethane thiol with TCIN, at the Nmr Centre, Griffith University, Brisbane. Noise decoupled spectra were determined in d^6-acetone.

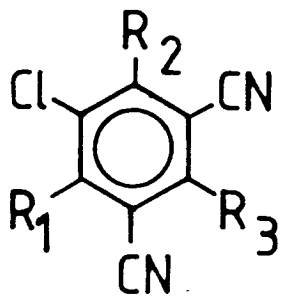
7.2.3 RESULTS

Formulae of the pesticide and its conjugates are shown in Fig. 7.7. The mass and FTIR spectra of the ethylmercaptan and mercaptoethanol model adducts are detailed in Table 7.2. In all cases, the M^+ ion, isotope and degradation patterns in the mass spectra confirmed the molecular formulae and the presence of thioether linked functional groups in positions formerly occupied by chlorine in TCIN.

N.m.r.(H) spectra for the ethylmercaptan conjugates did not indicate any differences in $-S-CH_2-$proton δ values between the three compounds. This indicated that the $-S-CH_2-$moiety was too far removed from the benzenoid nucleus to experience differences in electronic environment due to substitution position differences.

Details of the FTIR spectra for glutathione, TCIN, and G1 are shown in Table 7.3. All peaks are in agreement with the proposed structure of the adduct G1, although there is no information on the position of thiol-substitution. The N.m.r.(H) spectrum of G1 was essentially identical to that of glutathione except for the disappearance of the exchangeable SH proton peak, and some slight shift differences.

The infrared ArCN stretch vibration absorbance peak is weak in TCIN and even weaker in the thiol adducts. Using single scan I.R. spectroscopy, Vincent and Sisler (1982) could not discount CN loss on reaction with mercaptoethanol on the basis of the I.R. CN peaks they observed. FTIR spectroscopy allows greater peak resolution and enhancement. The CN signal was sharp and prominent in all TCIN conjugate analogue spectra observed in this work, even in tri-adduct



Compound	R_1	R_2	R_3
TCIN	Cl	Cl	Cl
DAC3701	OH	Cl	Cl
Sulphate ester	$OSO3^-K^+$	Cl	Cl
E 1	SCH_2CH_3	Cl	Cl
E 2	SCH_2CH_3	SCH_2CH_3	Cl
E 3	SCH_2CH_3	SCH_2CH_3	SCH_2CH_3
M 1	SCH_2CH_2OH	Cl	Cl
M 2	SCH_2CH_2OH	SCH_2CH_2OH	Cl

Fig. 7.7 Structures of TCIN and derivatives synthesized in this work. E = ethane thiol adducts; M = mercaptoethanol adducts

Table 7.2 Mass and FTIR spectral characteristics of model TCIN thioethers

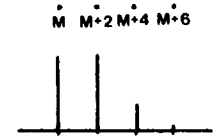
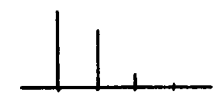
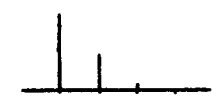
Compound	E.I. mass spectrum		Isotope pattern	FTIR spectrum	
	Main ion groups			Peaks (cm^{-1})	Group stretch
E 1	290 - 2	(mol. ion)		2855,2928	CH_2-CH_2
	262 - 4	(- CH_2=CH_2)		2236	ArCN
	226 - 8	(- Cl)		1618,1542	ArC=C
				1354	C-S
				724,739	C-Cl
E 2	316 - 8	(mol. ion)		2965,2927	CH_2-CH_3
	260 - 2	(- 2xCH_2=CH_2)		2235	ArCN
	226	(- Cl)		1618,1528	ArC=C
				1347	C-S
				715,768	C-Cl
E 3	342 - 4	(mol. ion)		2965,2927	CH_2-CH_3
	313 - 5	(- CH_2=CH_2)		2235	ArCN
	285 - 7	(- 2xCH_2=CH_2)		1617,1527	ArC=C
				1347	C-S
				715,768	C-Cl
M 1	306 - 8	(mol. ion)	As for E 1	-	-
	276 - 8	(- CH_2OH)		-	-
M 2	348 - 350	(mol. ion)		-	-
	288 - 290	(- 2xCH_2OH)		-	-
	274 - 6	(- CH_2)	As for E 2	-	-
	228	(- S-CH_2)		-	-
	239 - 240	(- Cl)		-	-

Table 7.3 FTIR spectral characteristics of TCIN, GSH, G 1 and derivatives

Compound	Peaks (cm^{-1})	Group designation	Peaks (cm^{-1})	Group designation
TCIN	2241,2234	ArCN str.		
	1549,1528,982	ArC=C str.	-	-
	739,756	C-Cl str.		
GSH	3347,3252	2^{o} amide str.	1562	NH_3^+ deformations
	3129,3024-2400	COOH str., $COO^- NH_3^+$	1537	amide II
	2906,2862	C-H str.	1414	$S-CH_2$
	2525	S-H str.	1414	$S-CH_2$
	1713	amide I	1395	SCH-H str.
	1663	amino acid I (COOH)	1352,1169	C-C bnd., str.
	1600	amino acid II(COO^-)	1352,1333,1074	C-S str.
G 1	3405,3265	2^{o} amide str.	1552,1455	amide II,ArC=C str.
	3123,3061-2400	COOH str.,$COO^- NH_3^+$	1384	C-C bnd.
	2235	ArCN str.	753	C-Cl str.
	1644,1606 br.	amide II, amino acid I, II		
GSH	3328,3309	2^{o} amide str.	1556	amide II
(N-TFA, nbutyl	2965,2939,2878	C-H str.	1361,1184	C-C bnd., str.
ester deriv.)	1723	amide I, ester C=O		
G 1	3333 br.	2^{o} amide str.	1558	amide II
(N-TFA, nbutyl	2964,2937,2877	C-H str.	1387,1183	C-C bnd. str.
ester deriv.)	1723	amide I, ester C=O		

compounds. This, along with mass spectral data, confirmed that CN loss does not occur in the thiol-TCIN reaction.

The mass spectra of glutathione and the mono-adduct G1, when subjected to C.I.(NH_3) were in agreement with regard to principal mass peaks at mass units 373 and 356, corresponding to loss of the CF_3 and n-butanyl fragments (with loss of the TCIN mo ty in G1) from the derivatized glutathione moiety, corresponding to fragmentations 1 and 2 in Fig. 7.8; and 186, corresponding to the glycine fragment with loss of CF_3 - fragmentations 2 and 3. In addition, derivatized G1 gave a C.I. spectrum with peaks at 672, corresponding to loss of the n-butanyl function from the molecular ion - fragmentation 1 (Fig. 7.8); and 561 and 577, corresponding to loss of the n-butanyl and glutamine functions - fragmentations 4 and 5. No molecular ion peak could be observed for either molecule.

The E.I. spectra of both compounds gave the same fragmentation pattern corresponding to complete fragmentation of the glutathione moiety. Principal peaks occurred at 254, corresponding to the derivatized glycine fragment (fragmentation 3, Fig. 7.8); 198, 180, corresponding to the subsequent loss of n-butanyl by fragmentation 4 and 1 respectively; 152, corresponding to subsequent loss of CO (fragmention 6) and 84, corresponding to subsequent loss of the CF_3 moiety (fragmentation 2). Peaks also occurred at 57, corresponding to loss of water from the n-butanyl fragment produced on fragmentation 1, and at 69 corresponding to the CF_3 moiety. The spectrum of derivatized GSH also gave a peak at 356 mass units corresponding either to loss of the glutamate fragment or of CF_3 and n-butanyl fragments from the molecular ion. No such peak occurred in the spectrum of G1. No molecular ions were obtained in any of the mass spectra. This is to be anticipated due to the high molecular-weight and low volatility of these polar compounds. Derivatization in the manner described substantially increased the volatility compared to the parent compounds, which gave no interpretable spectra on direct E.I. or NH_3 C.I..

The calculated aromatic carbon spectra for the four possible structures of E2 are shown in Table 7.4 along with the experimentally determined spectrum for E2. This confirms that the structure of E2 is that expected on the grounds of resonance considerations, namely that the di-adduct E2 is thiol substituted in the 4, 6 positions (Table 7.4, 1). By inference, the mono-adduct E1 must be thiol substituted at the

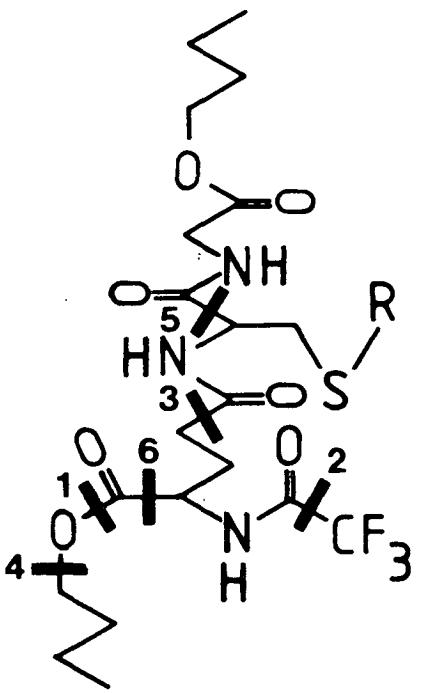
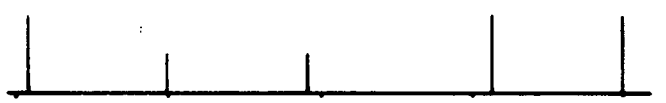
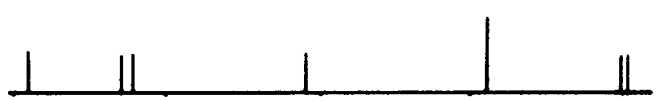
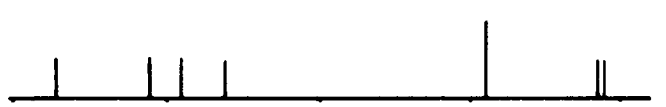
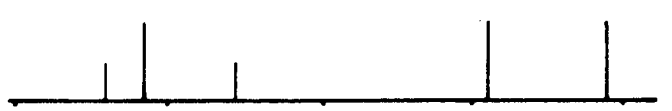
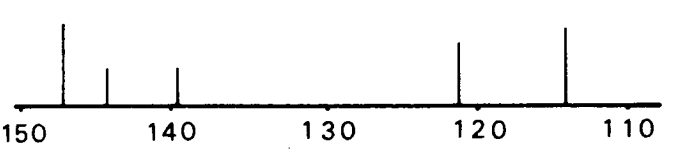


Fig. 7.8 Structure of n-TFA n-butyl ester derivative of GSH (R = H) and G1 (R = TCIN), with proposed mass spectral fragmentations.

Table 7.4 Predicted (P) and experimentally determined (D) C^{13} Nmr spectra of the aromatic carbon region for the TCIN thiol adduct E2

Thiol substitution pattern	Spectrum
P. I: 4, 6	
P. II: 2, 6	
P. III: 5, 6	
P. IV: 2, 5	
D. E 2: (4, 6)	

4 position, as expected. This firmly established the structures of the TCIN thiol adducts.

7.2.4 DISCUSSION

The thioether nature of the TCIN adducts is confirmed in this work, and a final confirmation was obtained on the positions of substitution of the multiple conjugates. It was considered that N.m.r.(H) evidence would go some way toward demonstrating the symmetry of the di-adduct and indicating the position of final substitution in the tri-adduct. However, the S-CH_2- groups in the ethanethioadducts showed no shift differences, even in the tri-adduct. Although no difference in shift would occur between the S-CH_2- functions in the symmetrical di-adduct, some signal splitting could be expected for the final substitution function in the triadduct due to a difference in chemical environment around the CN functions. It is possible that the thiophenolic or aryl-H compounds generated by reductive cleavage may show suitable differences in proton values, but they could not be generated in this work by Birch or Raney Nickel reduction. The ease of reductive breakdown into non-benzenoid fragments may be a product of the dinitrile substitution. No indication of substitution pattern occurs or would be expected in the M.S. or FTIR spectra.

The identity of glutathione bound to TCIN is established by:

a) the formation of the adduct in the presence of glutathione,

b) the ninhydrin reaction,

c) the presence of characteristic peaks for TCIN and glutathione in the FTIR spectrum, with the loss of the SH vibration,

d) the presence of characteristic peaks for TCIN and nTFA-nbutyl ester groups in the FTIR spectrum of the derivatized conjugate,

e) some peak matching between the mass spectra of the conjugate and glutathione,

f) characteristic peaks in the N.m.r.(H) for the glutathione moiety and predictable carbon shifts in the C^{13} N.m.r. spectrum of analogue adducts.

The use of a number of soft-ionization techniques for mass spectrometry has been recommended for biological compounds which decay on attempts at ionization by E.I. and C.I. techniques, as occurred here. They are field desorption (Przybylski et al., 1979), fast atom bombardment (Barber et al., 1981), and electrohydrodynamic ionization (Stimpson and Evans, 1978). These techniques were unavailable for use in this work.

Although CN I.R. stretching frequencies are generally regarded by chemists as being relatively invariable, correlations between the small shifts in frequencies of benzonitrile CN stretching with Hammet functions, on changing benzene functional groups have been made (Bellamy, 1980). Small wavelength changes with concentration of the order of 4 cm^{-1} also occur, due to the ability of the nitrile group to associate. Some information on changes in CN polarity due to the substitution differences in benzonitriles can be gained by studying the highly variable intensity of the relatively weaker conjugated aromatic nitrile group stretch frequencies (Bellamy, 1980).

Deady et al. (1975) gave the following CN stretch frequencies and intensities for ortho-substituted benzonitriles:

o-substituent	V_{CN}	A
NH_2	2219.6	1680
OMe	2229.3	1040
Cl	2235.5	430

A similar but minor effect is noted for meta-substitution. There is, therefore, a slight downward wavelength shift with increasing electron donating ability of an ortho substituent. Such a shift in the CN stretch frequency peak is observed, from 2241 cm^{-1} in TCIN, to 2236 in the E1 model adduct, and to 2235 in E2 and E3 (Table 7.2 and 7.3). It is probable that these differences are due to the exchange of the strongly electron-withdrawing Cl for the elctron rich sulphur thioether function. It is not possible, however, to make deductions about the position of exchange from this data.

Infrared signal intensities increase with electron donating ability of the ortho-substituent. The signal intensities of solid KBr preparations of the same mass content, in the spectra of TCIN, E1, E2 and E3 are 0.185, 0.034, 0.088 and 0.075 absorbance units respectively. If intensity variations, which are also concentration dependent (Deady et al., 1975), in the CN stretch signals were only due to the electron donating ability of ortho-subsituents, the expected order would be: TCIN > E1 > E2 > E3. The experimental order does not agree exactly with this, and consequently no structural information can be gained, although the fact that the model adduct compounds have lower CN intensities is as expected. It is also of interest to note that the CN stretch signal intensity for G1 was 0.06 units, much lower than for TCIN.

The presence of strong nitrile peaks in the IR spectrum in all thiol adducts, as well as the mass spectral data, confirm that the nitrile function in TCIN is stable to nucleophilic attack. This is not surprising since the CN π bond system is effectively linked with and delocalized by the π electron system of the benzene ring, and is subsequently stabilised by it. Silver et al. (1982) investigated the effect of structural factors on the metabolism of organic nitriles to cyanide in vivo. The metabolic processes available to unsaturated nitrile compounds leading to the release of cyanide, Michael addition at the α, β-double bond, epoxide formation and cleavage and hydroxylation at the β-carbon, are not available to TCIN due to steric and aromatic stability effects.

In conclusion, this work finally confirms the exact structures of TCIN thiol adducts. The structures agree with those first postulated by Vincent and Sisler (1968), and with those expected from resonance considerations. Since thiol binding is favourable in a defined sequence of sites on TCIN, such binding by protein thiol functions would be expected to be limited by steric constraints, as well as by the nature of the protein thiol itself. This ability of TCIN to bind with thiol proteins is investigated in the subsequent section, and in further chapters.

7.3 PROTEIN BINDING OF TCIN

7.3.1 INTRODUCTION

Protein binding and enzyme inhibition by drugs or xenobiotics is well demonstrated in toxicology and pharmacology for a large number of compounds (La Du et al., 1972), and are often the cause of biological activity in such compounds. TCIN was shown to bind to glyceraldehyde 3-phosphate dehydrogenase, and nuclear proteins in vitro by Long and Siegel (1975) and Rosanoff and Siegel (1981), causing inhibition of the former enzyme. The previous chapter showed that protein binding appeared to be occurring in TCAA protein precipates. It was decided to investigate the degree of protein binding in liver and bile in more detail.

Metallothioneins are low-molecular weight, cysteine-rich binding proteins capable of binding to a variety of metals including cadmium, copper, zinc and mercury by liganding of the cysteine thiol residues. Methallothioneins have been well established and researched in rats, but their physiological significance is still unclear (Oh et al., 1978). They are readily induced by exposure to the above metals, and this behaviour is interpreted as playing a projective detoxication role against deleterious metal binding. This scenario has also been invoked in fish, where metallothionein induction has been established, and where it plays a significant role in metal dynamics during exposure (Reichert et al., 1979; Kito et al., 1982 a, b, c), although note the cautionary work of Thomas et al. (1983).

Since the thiol-binding ability of TCIN is well established, and it appears that TCIN binds to hepatic protein in S. gairdneri, then metallothionein may possibly play a significant role in TCIN detoxication, by the alkylation of cysteine residues by TCIN. It was decided to investigate the relationship between TCIN and metallothionein in conjunction with zinc exposure in order to also investigate the possibility of in vivo TCIN binding to metallothionein when metallothionein has been induced by zinc.

7.3.2 MATERIALS AND METHODS

General protein binding

Aliquots of 0.40 ml liver cytosol (prepared as described previously, 7.1.2), from unexposed fish, were diluted with 2 ml 0.1 M Tris - HCl buffer (pH 8.3), and incubated with 8.4 ul of a C^{14}-TCIN/ acetone stock, giving a final TCIN concentration of 0.5 ug/ml. Three such preparations each were incubated at 20° C for 2, 6, 20 and 120 min. They were then mixed with 0.50 ml 25% TCAA and centrifuged at 2000 g for 20 min. The pellets were resuspended in fresh buffer, recentrifuged, washed three times with acetone (2 ml), with recentrifugation, and digested with Soluene. All digests were radioassayed as described previously (7.1.2).

Liver and bile protein binding

Sephadex G75 gel column chromatography was performed on liver cytosol and fresh bile of S. gairdneri exposed to 10 ug/l C^{14}-TCIN for 96 h, as described in 7.1.2.

TCIN - zinc co-induction experiment

Five groups of four S. gairdneri (mean weight 12.0 g) were each exposed to the following conditions, at 15° C : 0.36 mg/l zinc (as zinc sulphate, dissolved in tank water) for 96 h, then 10 ug/l C^{14}-TCIN (activity 6065 dpm/ug) for 60 h; 0.36 mg/l zinc for 96 h, then 0.36 mg/l zinc and 10 ug/l C^{14}-TCIN for 60 h; 0.36 mg/l zinc for 96 h, then uncontaminated water for 60 h; 0.36 mg/l zinc for 96 h; uncontaminated water (acetone carrier only) for 156 h. Since acetone carrier was used in each pesticide treatment, it was added to all other treatments at the same concentration. Exposures were carried out in 26 l aerated tanks, with daily change of water.

At the end of exposure periods, fish were killed (tricaine methanosulphonate bath), and the livers excised, pooled, homogenised in 8 vol. 0.1 M Tris - HCl (pH 8.3) and centrifuged at 30,000 g for 1 h. The supernatants were subjected to gel column chromatography as described in 7.1.2. 1.3 ml fractions were collected, radioassayed as described (7.1.2), and analysed for zinc by direct aspiration atomic absorption spectroscopy on a Varian Techtron spectrometer. A standard curve for zinc analyses was prepared in the range 0 - 10 mg/l zinc, using zinc sulphate solutions.

7.3.3 RESULTS

TCIN binding by protein in hepatic cytosol preparations was confirmed (Table 7.5), and showed a similar trend of initial increase then a plateau with time as occurred during exposure of fish in the radiolabel exposure experiment described earlier (Chapter 6).

Protein bound TCIN in bile comprised 1% of the total radiolabelled material (Fig. 7.9, 1), with 99% occurring in the low molecular weight, non-protein fractions. In the liver (Fig. 7.9, 2) 51% of the labelled material was associated with high molecular weight protein fractions and 49% occurred in the low-molecular weight non-protein fractions.

Fig. 7.10 shows gel column fractionation profiles for the zinc - TCIN co-induction experiment. Peak molecular weight estimates are given in Table 7.6. Control fish showed low zinc levels for all protein fractions with a peak in the high molecular weight fractions (Fig. 7.10 a). Exposure to 0.36 mg/l Zn for 96 h showed a general increase in zinc levels especially in the high molecular weight (HMW) fractions, with significantly increased Zn binding at 25 100 and 20 900 MW. No increase occurred in Zn levels in the 6000 - 12000 MW range (Fig. 7.10, b). Following depuration for 60 h in fresh water, the HMW fractions zinc levels increased by a factor of 2. The two peaks observed in Fig. 7.10 b were not evident (Fig. 7.10 c). When 96 h exposure to 0.36 mg/l was followed by 60 h exposure to 10 ug/l C^{14}-TCIN, the zinc profile was identical in concentration and molecular weight distribution to that after 60 h depuration (Fig. 7.10 d). C^{14}-TCIN binding occurred predominantly in the HMW region with two peaks at 79000 and 56000 MW. On following 96 h exposure to 0.36 mg/l Zn with 60 h exposure to 0.36 mg/l Zn and 10 ug/l C^{14}-TCIN, the Zn profile was similar to that shown in Fig. 7.10 b, although with higher overall Zn levels and the addition of a new Zn peak at 8000 MW corresponding to metallothionein (MT) (Fig. 7.10 e). The HMW Zn content increased only marginally compared to the 96 h exposure period. The predominant increase in Zn binding occurred in the two 23400 and 19500 MW peaks and the MT peak. C^{14}-TCIN binding was, however, much lower than in the latter. No increase in C^{14}-TCIN binding could be observed in the MT region (Fig. 7.10 e).

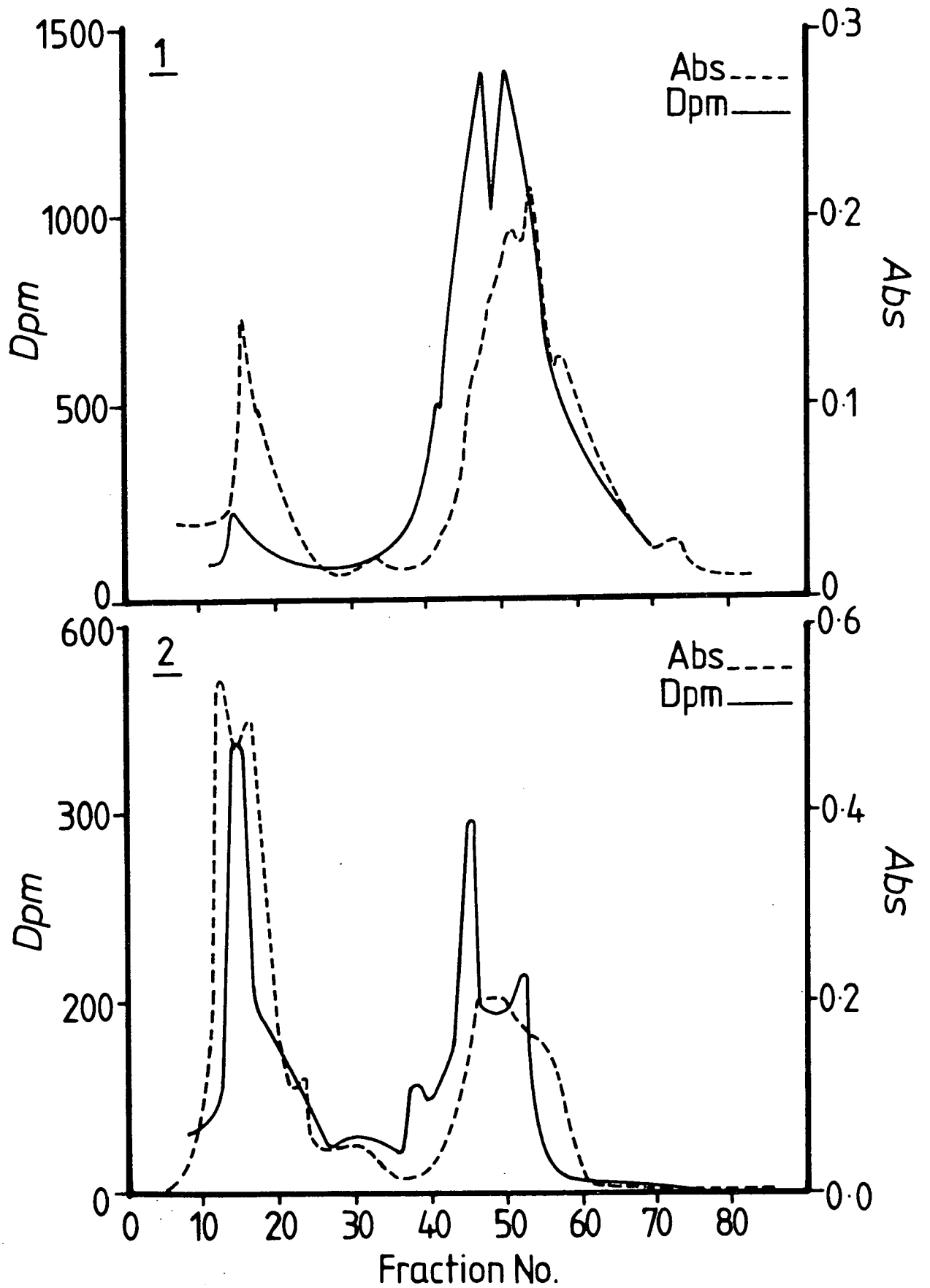
Table 7.5 Protein binding of C^{14}-TCIN in liver cytosol

Time of exposure (min)	ug TCIN bound per g liver
25	4.457
60	4.535
120	6.447

Table 7.6 Molecular weights of binding peaks for Zn and C^{14}-TCIN in hepatic cytosol of S. gairdneri. * = MT fraction

Treatment	Assay	Molecular weights
0.36 mg/l Zn, 96 h	Zn	135000, 53700, 25100, 20900
0.36 mg/l Zn, 96 h; freshwater, 60 h	Zn	145000, 70800, 4300
0.36 mg/l Zn, 96 h;	Zn	151400, 63100
10 ug/l C^{14}-TCIN, 60 h	C^{14}-TCIN	126000, 79400, 56200
0.36 mg/l Zn, 96 h;	Zn	95500, 23400, 19500, 8300*
0.36 mg/l Zn, 10 ug/l C^{14}-TCIN, 60 h	C^{14}-TCIN	125900, 66100, 47900
10 ug/l C^{14}-TCIN	C^{14}-TCIN	120200, $<$ 1000, $\ll$ 1000
Unexposed	Zn	166000, 66100, 10000

Fig. 7.9 Gel column chromatogram profiles of S. gairdneri bile (1) and hepatic cytosol (2) after 4 days exposure to 10 ug/l C^{14}-TCIN.
Fractions 10 - 25 = high molecular weight proteins.
Fractions 40 - 60 = non-protein, low molecular weight.


1
Abs
Dpm
1500
1000
500
0
0·3
0·2
0·1
0
Dpm
Abs
2
Abs
Dpm
600
300
200
0
0·6
0·4
0·2
0·0
Dpm
Abs
0
10
20
30
40
50
60
70
80
Fraction No.

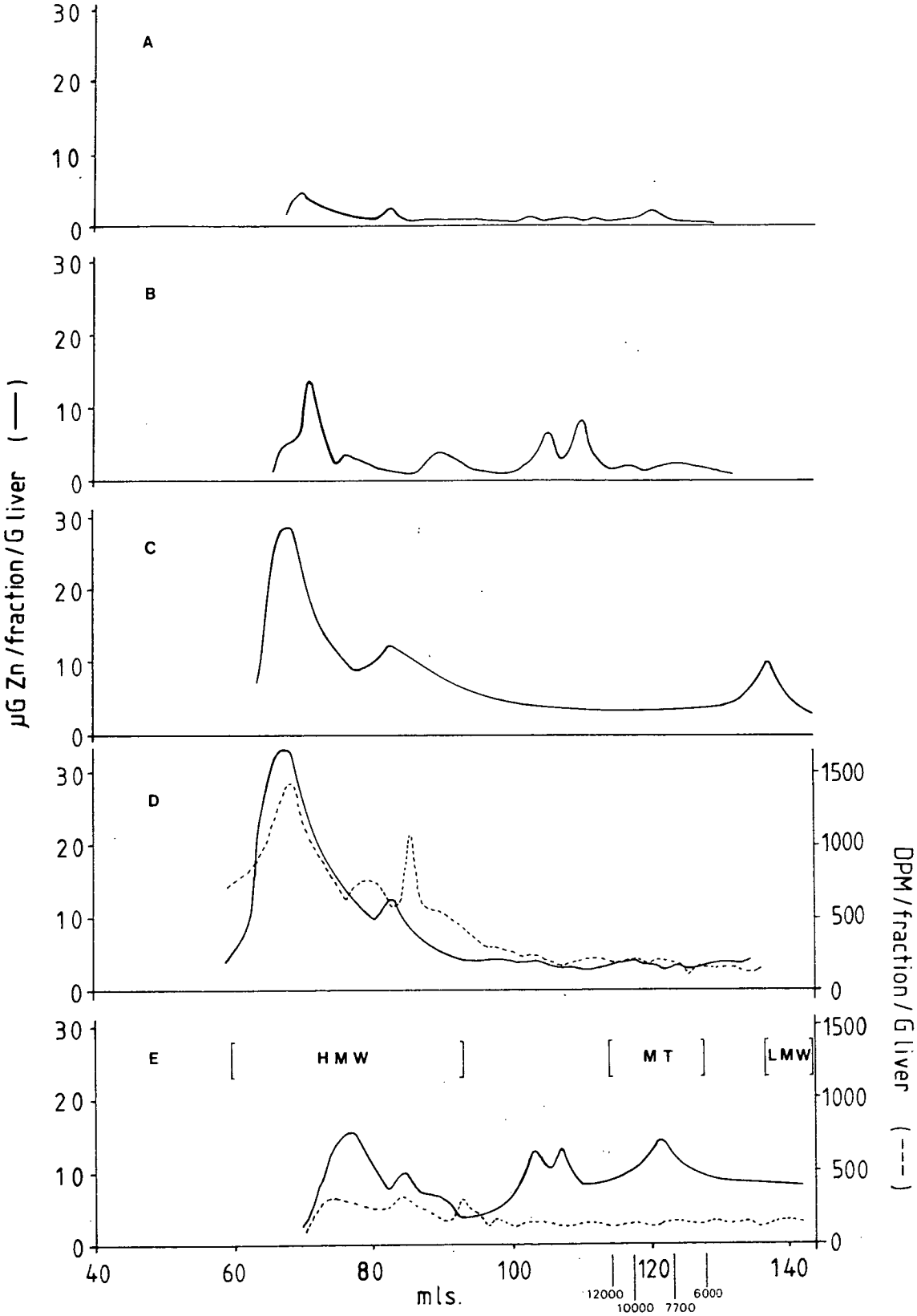


Fig. 7.10 Gel column chromatograms of S. gairdneri hepatic cytosol proteins. **A**: control fish. **B**: Fish exposed to 0.36 mg/l Zn (96 h). **C**: Fish exposed to 0.36 mg/l Zn (96 h), uncontaminated water (60 **D**: Fish exposed to 0.36 mg/l Zn (96 h), 10 ug/l C^{14}-TCIN (60 h).**E**: Fish exposed to 0.36 mg/l Zn (96 h), 0.36 mg/l Zn and 10 ug/l C^{14}-TCIN (60 h). See text for details.

7.3.4 DISCUSSION

Protein binding of TCIN in the liver may be a major cause of toxicosis. The plateau of protein-bound TCIN levels after 20 min in assay preparations indicates possible competition with the glutathione conjugation reaction. The different ratios of bound to low-molecular weight labelled residues in hepatic cytosol and bile reflects the different pharmacokinetic roles of the liver and gall bladder. The liver is regarded as the primary site of detoxication reactions including glutathione conjugations (Arias et al., 1982). The products of such reactions are passed into the bile, which contains little in the way of protein. Consequently, the organ concentration of low-molecular weight TCIN metabolites is lower relative to protein bound label, which may be regarded as a relatively static sink of TCIN residues, in the liver than in the bile. There is some protein binding in the bile. It is not clear whether this is due to in-bile binding or that the TCIN-bound proteins are passed out into the bile to be excreted.

4-Ipomeanol is a microsomally activated alkylating agent and cytotoxin in rats, which forms glutathione conjugates after metabolic activation (Buckpitt and Boyd, 1980). The activated form binds to macromolecules, and apparently exerts its cytotoxicity in this manner. Glutathione aparently competes effectively for this activated form when at high enough concentrations, converting it to conjugate metabolites. It is possible that TCIN follows this scenario, although without requiring or being suitable for structural activation. Pesticides are well known to cause enzymatic inhibition (Whitmore and Hodges, 1978) leading to toxicosis, and this possibility is investigated in subsequent chapters.

The C^{14}-TCIN hepatic protein binding profile shown in Fig. 7.9 showed no peak in C^{14}-TCIN levels in the 6000 - 12000 MW region, usually associated with metallothionein proteins (MT). This indicates that MT binding was not a significant detoxication route in S. gairdneri. It is known in S. gairdneri from the work of Olson et al. (1978) who exposed fish to radiolabelled MeHgCl, and from the work of Ley et al. (1983) who injected S. gairdneri with $ZnCl_2$. Both groups

isolated a hepatic MT fraction, of 6000 - 10000 and 7700 MW respectively. Olson et al. (1978) found that MeHg was predominently bound to HMW fractions but that MT was the next most important mercury pool, being most significant in the liver. In contrast Ley et al. (1983) reported that the major hepatic Zn pool was in the MT fraction with small Zn peaks in the HMW fraction and at two intermediate molecular weights. The predominance of the MT fraction when compared to the work of Olson et al. (1978) and the results obtained here, is probably a function of the administration route and the high level of the metal dose used by Ley et al. (1983).

That S. gairdneri MT has a high binding ability is emphasised by the occurrence of a high level (25%) of cysteine residues in the protein (Ley et al., 1983). It appears, however, that despite being rich in thiol groups it does not bind C^{14}-TCIN in vivo either on exposure to C^{14}-TCIN alone, after or with exposure to 0.7 toxic units of Zn. It is both incapable of binding to MT when present (Fig. 7.9 e), or inducing it (Fig. 7.10 d). However, the metabolism of Zn and C^{14}-TCIN by S. gairdneri are not entirely unrelated. It is not surprising that the hepatic protein-binding profile of Zn after exposure and depuration is not affected by exposure to C^{14}-TCIN (Fig. 7.10 c,d), since both the dose and protein-bound level of C^{14}-TCIN were much lower than those of Zn. However, it appears that continued exposure to Zn in the presence of C^{14}-TCIN does suppress hepatic protein-binding of C^{14}-TCIN. Since the height ratios and MW's of the C^{14} -TCIN bound peaks are the same in Figs. 7.10 d and 7.10 e, this process is not selective, but rather occurs across the entire protein molecular weight range. Thus, there appears to be a general mass-inhibition of TCIN protein binding by Zn on co-exposure. This may be due to competition for thiol binding sites which, despite obvious binding preferences in Zn (Fig. 7.10 e) due to the relatively low C^{14}-TCIN binding levels is non-selective for protein molecular weight. It would be of interest, in this light, to compare LC50 responses for S. gairdneri to TCIN - Zn mixtures. If TCIN - Zn LC50's were higher than for TCIN alone, then more weight may be given to the hypothesis that TCIN toxicity is due to general protein binding.

Wong and Klaassen (1981) showed that diethyl maleate and bromobenzene caused a decrease in glutathione levels in rats, followed

by a doubling in the hepatic metallothionein content, in contrast to Zn, which increased MT but did not decrease glutathione levels. Cagen and Klaassen (1980) established that the glutathione-depleting agents bromobenzene and chloroform did not bind to metallothionein after zinc pretreatment in rats. They did, however, bind in vitro preparations. These workers also established that MT was much harder for another alkylating agent, iodoacetate, to bind to than glutathione, requiring 10 times higher concentrations of iodoacetate to produce the same degree of depletion. This was related to a protective role of glutathione, and led to the suggestion that MT binding may only act as a significant detoxication route when glutathione levels are low. The above results are entirely consistent with those obtained here for TCIN binding in S. gairdneri.

Toby et al. (1982) investigated the survival of hamster cell lines of differing ability to induce MT, on exposure to the glutathione depleting alkylating agent, iodoacetate, after preexposure to zinc. There was no correlation between survival and ability to induce MT, confirming the "somewhat surprising" insignificant nature of the MT - alkylating agent interaction and its role in detoxication. However, they did find a significant correlation between zinc exposure concentrations and survival, indicating a protective role for zinc. Since the toxicity of iodoacetate is believed to be due to its protein binding ability, the apparent role of zinc is the same as occurs in this work. Toby et al. (1982) suggest that Zn exposure may cause an increase in GSH, G S-transferase activity, levels of other binding proteins, or DNA repair capacity. In the present work on S. gairdneri, Zn protection against TCIN protein binding may be due to one of the first three reasons. Wong and Klaassen (1981) showed that Zn caused an increase in GSH levels in rats prior to metallothionein induction. Certainly, an increase in GSH levels would increase the ability of S. gairdneri to detoxify TCIN and may relieve any toxicosis caused by GSH depletion.

TCIN appears to be a powerful alkylating agent but shows no binding behaviour towards MT in vivo. Oh et al. (1978) found that a variety of environmental stresses, including administration of the glutathione - depleting hepatotoxin, carbon tetrachloride, caused

induction of MT in rats. TCIN does not, however, appear to induce MT in S. gairdneri. To what extent glutathione levels are affected by TCIN exposure and whether glutathione acts in a protective role toward MT and other proteins against TCIN binding is examined in Chapter 9.

CHAPTER 8

CATALYSIS OF THE TCIN-GLUTATHIONE REACTION : GLUTATHIONE S-TRANSFERASES

8.1 CATALYSIS OF THE TCIN-GSH REACTION

8.1.1 INTRODUCTION

As already discussed in Chapter 6, the rate of accumulation of the C^{14}-TCIN metabolites in the bile of Salmo gairdneri was high, and this suggested the action of a catalyst in the conjugation of TCIN and glutathione (GSH). This was confirmed by the observation that the reaction between TCIN and GSH is slow at physiological pH values (Chapter 7). Catalysis of the reaction of GSH with electrophiles is known to be carried out by the glutathione S-transferase (GST) group of enzymes which have been well studied in mammals, where the primary role appears to be one of detoxication (Habig and Jakoby, 1981). It was considered relevant to establish whether the reaction between TCIN and GSH was catalyzed by any cytosolic enzymes in various organs of S. gairdneri.

The development of an assay technique with TCIN was investigated. 1-chloro-2,4-dinitrobenzene (CDNB) is used as a substrate for nearly all studies of the GST enzymes due to its ability to demonstrate a spectral shift on conjugation with glutathione from 247 nm to 340 nm, with a relatively high ϵ value of 9.6 units/mM/cm (Fig. 8.1). Therefore, a rate of increase in absorbance of CDNB-GSH enzyme assay mixture at 340 nm can be converted to GST enzyme activity. However, the mono-glutathione conjugate of TCIN, G1, shows only a marginal spectral shift from 235 nm in TCIN to 230 nm (Fig. 5.1). This, despite a high ϵ value, precludes a spectrophotometric assay.

A convenient means found for assaying catalytic ability toward TCIN was to differentiate the reactant and products on the basis of their polarity using the radiolabelled C^{14}-TCIN as a subtrate enabling quantification of conversion. TCIN is readily extracted from water with

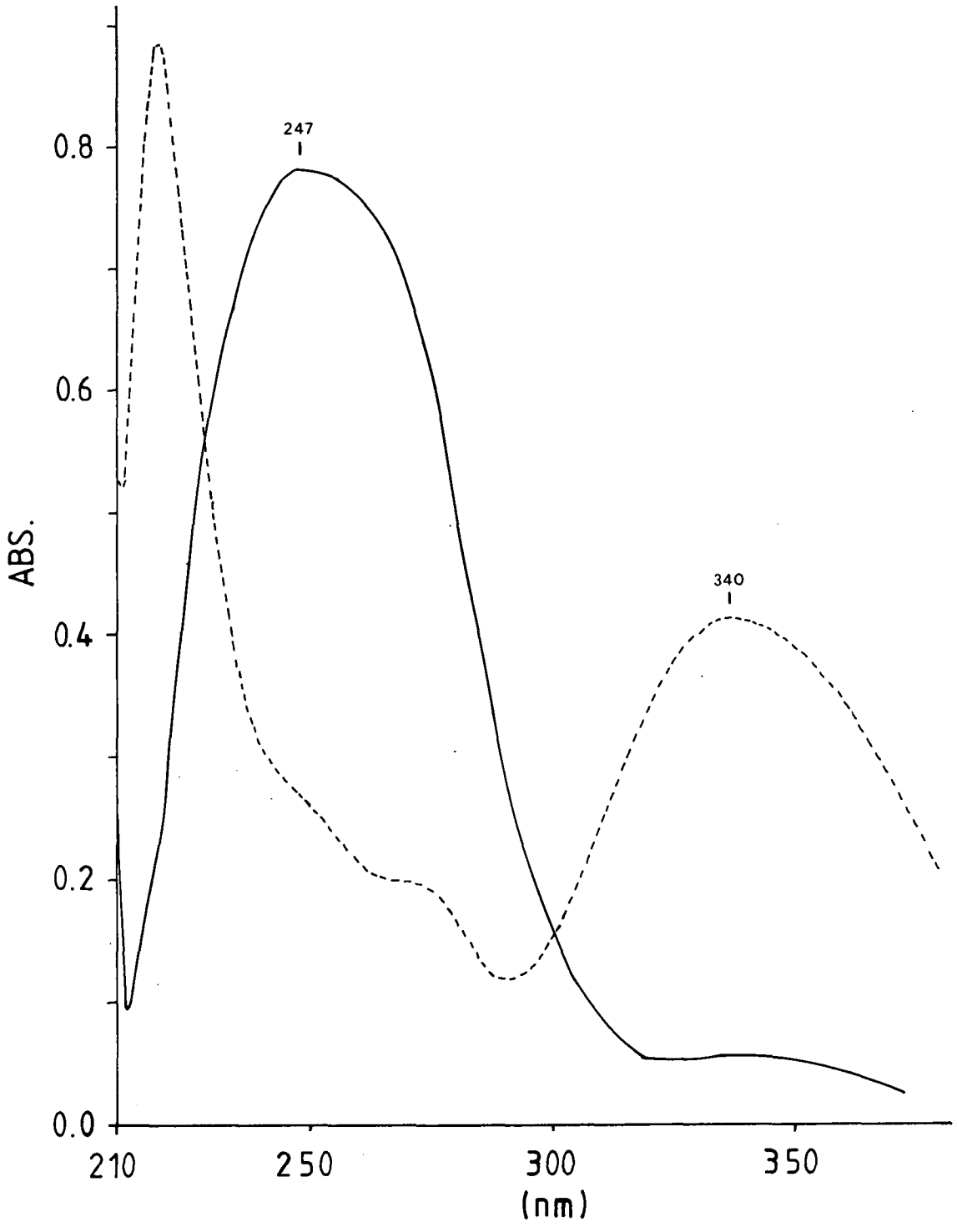


Fig. 8.1 Ultraviolet spectra of 1-chloro-2,3-dinitrobenzene, CDNB (——), and its glutathione conjugate (---).
0.1 M Tris-HCl(pH8.3); 30 ug/ml.

hexane, whereas G1 is entirely water soluble. Hexane and water phases could, therefore, be radiocounted after extraction of the protein-denatured assay mixture in order to calculate the levels of C^{14}-TCIN and polar TCIN-GSH conjugates remaining after reaction. This method is suitable only if assay incubation times are matched with the initial linear phase of the assay concentration curves.

The ability of cytosolic preparations of a number of organs of Salmo gairdneri to catalyze the TCIN-GSH reaction was investigated by the above method, and the pH dependence of the reaction rate in the presence of liver cytosol was measured.

8.1.2 MATERIALS AND METHODS

Sample preparation and assays

1. Organ cytosol preparation

Salmo gairdneri (8 ± 3 g) were supplied by Sevrup Fisheries Pty. Ltd. and acclimated for 10 days in the flow-through system with daily feeding as previously described (3.1.2). Fish were killed (tricaine methanosulphonate bath), and dissected immediately.

Preweighed organs were homogenized in 8 vol. cold 0.01 M Tris-HCl (pH 8.3) buffer, 1 mM in GSH, henceforward called buffer A. Homogenates were centrifuged at 30,000 g for 1.5 h (4° C) and used either immediately or after snap-freezing and storage in liquid nitrogen.

2. C^{14}-TCIN reaction assay. Standard procedure.

Aliquots of cytosol preparations, typically 100-200 ul, were incubated at 20° C in 1-2 ml buffer A, for either 1, 2 or 3 min after addition of 5-10 ul of a 1000 mg/l C^{14}-TCIN-acetone stock solution. Final assay total activity was 5000-8000 dpm. Cytosol-less controls were always run with each assay batch.

Reactions were stopped by addition of 0.5 ml 25% trichloroacetic acid solution. Solutions were then centrifuged at 2000 g for 20 min. The supernatants were subsequently extracted with hexane fraction (2 : 1) with magnetic stirring for 20 min (efficiency $\geq$ 98% by internal standards) and aliquots of the aqueous and hexane layers were radiocounted. When required, protein precipitate pellets were resuspended in fresh buffer, recentrifuged, acetone washed (2 x 5 ml) and

recentrifuged prior to digestion (Soluene $^{(R)}$) and radiocounting. All radiocounting was performed in 5 ml Dimilume - 30 (Packard) as described previously (Chapter 7).

All cytosol assay counts were corrected for the cytosol-less blank reaction after quench and background correction.

Confirmation of catalysis

The time-course of the TCIN-GSH reaction was followed in the presence of liver cytosol and fresh bile (100 ul each) and compared to the blank reaction.

Organ differences

TCIN-GSH reaction rates were determined for liver, bile, kidney, spleen and hind-gut caecae.

pH dependence

The standard assay method was used with liver cytosol preparations to measure the reaction rate over a range of buffer pH values.

8.1.3 RESULTS

The time-course of typical reaction sequences for TCIN and GSH in the presence of hepatic cytosol and bile are shown in Fig. 8.2, and can be compared with the reaction performed in buffer only. Catalysis occurred in the presence of hepatic cytosol, but not with bile. The initial rates of production of TCIN polar metabolites were increased by a factor of 10-20 by hepatic cytosol in assay mixtures.

Comparison of organ activities in S. gairdneri toward TCIN, given in Table 8.1, revealed that they decreased in the order:

$$\text{liver} > \text{kidney} \gg \text{spleen} > \text{caecae} > \text{bile} \cong 0.$$

The pH dependent activity profile of S. gairdneri hepatic cytosol toward C^{14}-TCIN is shown in Fig. 8.3, along with the uncatalyzed reaction. Peak activity occurred around pH 8, consistent with the choice of assay buffer pH and previous observations of Nimmo et al. (1972). Non-enzymic reactivity of TCIN and GSH increased with pH, consistent with the higher reactivity of the GS^- anion (Jakoby et al., 1976).

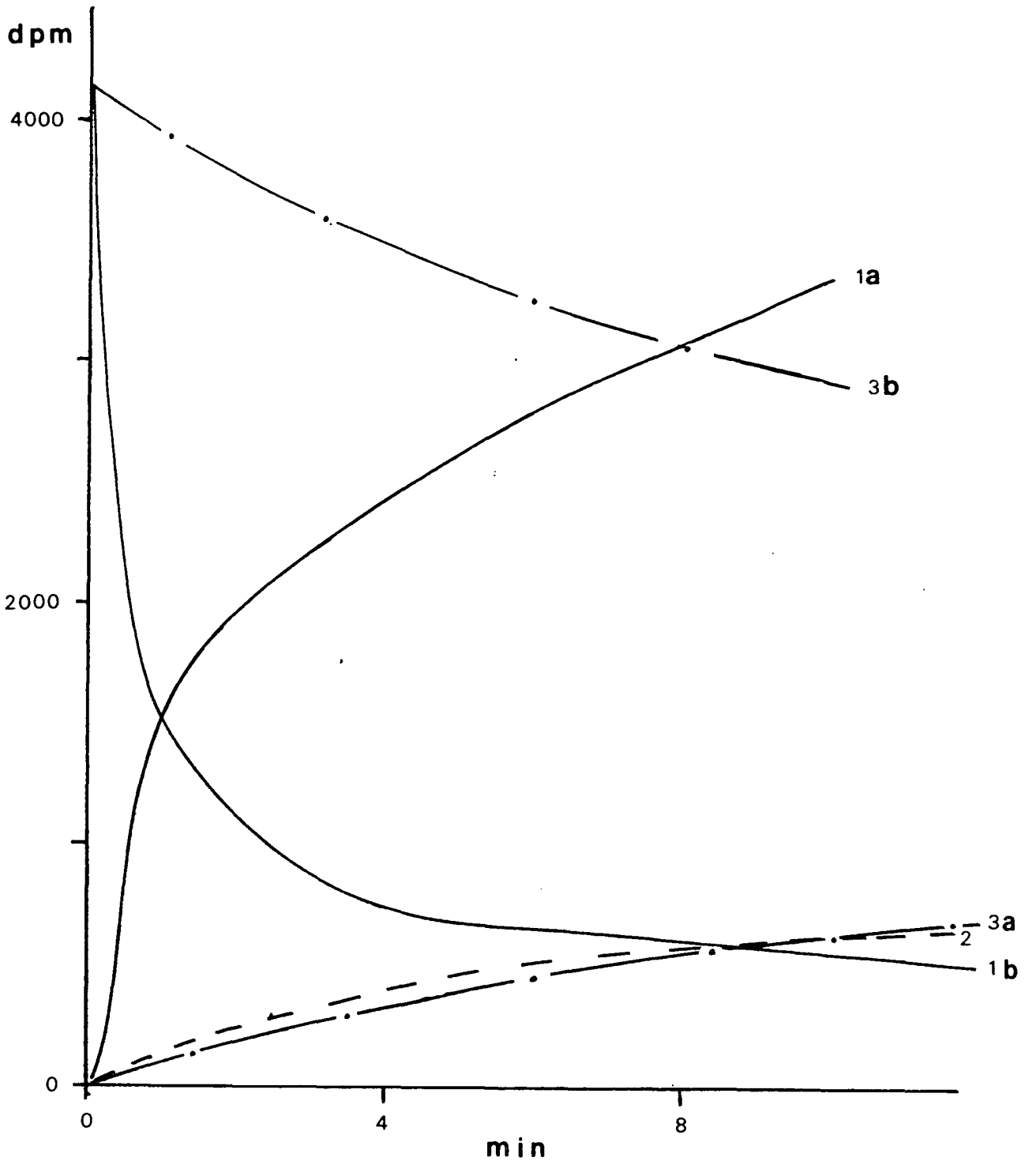


Fig. 8.2 Typical course of C^{14}-TCIN reaction with GSH in the presence of hepatic cytosol (1) and bile (2) from S. gairdneri.

1b = decrease of C^{14}-TCIN levels and 1a = increase in polar conjugate levels, both in the presence of hepatic cytosol.

3b = decrease of C^{14}-TCIN levels and 3a = increase of polar conjugate levels, both in the control assay.

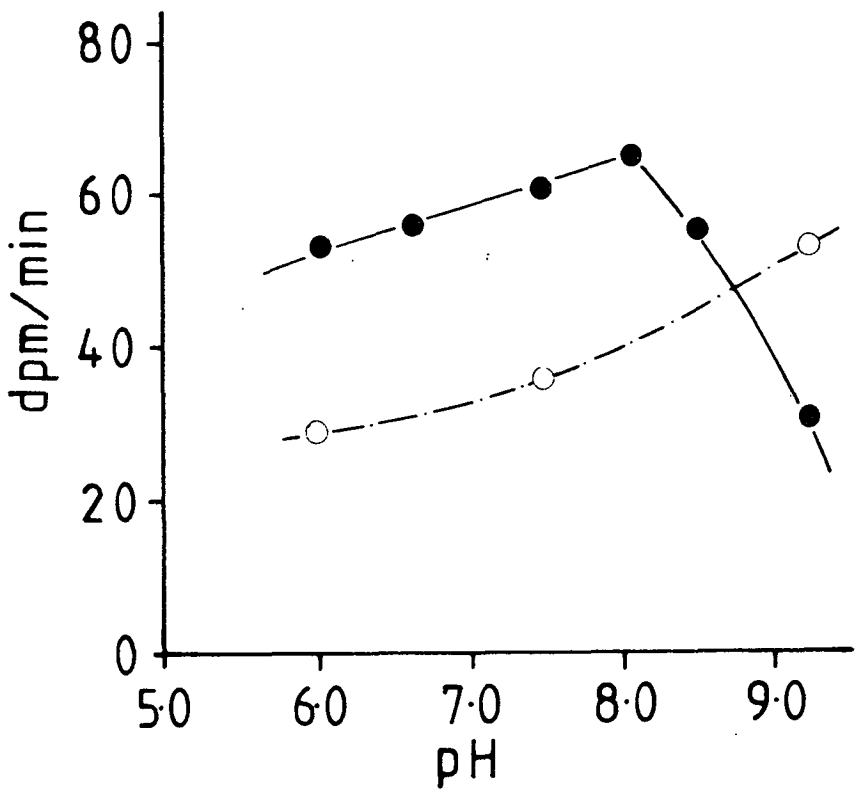


Fig. 8.3 pH - activity profile for S. gairdneri GST (●), and pH dependence of nonenzymatic reaction (○), plotted as production of aqueous metabolites under standard assay conditions (C^{14}-TCIN as substrate, 1 mM GSH, 20° C).

Table 8.1 Activities of G S-transferase toward C^{14}-TCIN in various organs of S. gairdneri

Organ	Activity
liver	45.75
kidney	32.68
spleen	1.12
bile	0.00

Table 8.2 Molecular weights of G S-transferase enzymes estimated by gel chromatography

Species	Substrate	
	C^{14}-TCIN	CDNB
S. gairdneri	60,300	45,000
S. trutta	56,200	42,200
G. maculatus	40,000	35,500
G. truttaceus	42,000	35,500
G. auratus	42,700	35,300

8.1.4 DISCUSSION

It appears that catalysis of the TCIN-GSH reaction is important in the liver, kidney and caecae of Salmo gairdneri. The liver is well established as the primary site of detoxication reactions for phase I and phase II transformations in mammals (Arias et al., 1982) and it appears that a similar picture occurs in fish (Chambers and Yarbrough, 1976; Khan et al., 1979; Gregus et al., 1983). Despite the fact that Hirata and Takashashi (1981) showed that biliary enzymes degrade glutathione conjugates and the coroboration of that finding here (Chapter 7), it appears that Salmo gairdneri bile contains no enzymic ability to convert TCIN to polar conjugates. Bauermeister et al. (1983) recently reported on the organ distribution of G S-transferase activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in Salmo gairdneri. The results obtained here are consistent with the order of organ activities they obtained. Comparison of absolute activity values is not possible due to the difference in assay concentrations of the two electrophilic substrates, TCIN concentrations being limited to less than 2.25 uM.

The pH optimum obtained for Salmo gairdneri activity toward TCIN was 8.1, and this was consistent with pH optima typical for G S-transferase enzymes examined in rats (Booth et al., 1962; Habig et al., 1974). Nimmo et al. (1979) performed G S-transferase assays with CDNB at pH 6.5 since they considered the non-enzymic reaction rate at higher pH's to be 'considerable' and they did not attempt to find the optimum enzyme pH. The reaction rate of TCIN with GSH increased with pH (Fig. 8.3) but can be substracted from the total rate in assays. Subsequently all assays were performed near the optimum pH, and corrections made for the non-catalyzed reaction. Studies of this enzyme activity were performed exclusively with cytosol preparations owing to the universal finding that the majority of G S-transferase activity is located in the soluble fraction, a typical example for hepatic cytosol being 87% of the total (Booth et al., 1982).

8.2 CHARACTERISATION OF THE G S-TRANSFERASE ENZYME ACTIVITY

8.2.1 INTRODUCTION

Glutathione S-transferases are enzymes which catalyze the reaction of glutathione (GSH) with compounds possessing a sufficiently reactive electrophilic centre. They also have a high affinity for binding many exogenous and endogenous substrates. They are medium weight enzymes with molecular weights of 30000 - 60000. They generally consist of two subunits of roughly equal molecular weight. One of these subunits possesses the ability to bind GSH, whilst the other binds the electrophilic substrate (Reed and Beatty, 1980).

There are a number of GST isozymes, each of which displays differences in activity toward a number of substrates. These include exogenous electrophiles, such as aryl halogens, nitroso compounds, aryl and alkyl nitro compounds and thiocyanates, endogenous compounds such as prostaglandin A, and endogenously generated activated electrophiles such as aryl epoxides (Jakoby et al., 1976). Gregus et al. (1983) demonstrated a remarkable variation in the activity of G S-transferases in Salmo gairdneri toward six different substrates. It was considered appropriate, therefore, that in a study investigating the possibility of GST mediation of TCIN metabolism, that C^{14}-TCIN be used as the primary substrate, and not the standard reference substrate CDNB, which was, however, used for the purpose of comparison.

Since catalysis of the TCIN-GSH reaction by hepatic cytosol was confirmed in Salmo gairdneri it was decided to investigate the following questions as part of an investigation into the role of enzymes in the toxic action of TCIN: whether the properties of the enzyme(s) were consistent with them being G S-transferase, whether the Galaxiidae also have this enzyme activity toward TCIN, how the properties of the fish enzymes compare between species, and whether TCIN is a substrate for a GST different from that with highest activity toward CDNB.

Gel column chromatography allows separation of proteins according to their molecular weight on passing the solution through a dextran gel of appropriate "molecular sieve" pore size to effect

separation of the proteins in the desired molecular weight range (Determann, 1968). Provided accurate pre- or co-calibration of the column is performed using proteins of known molecular weight, the linear dependence of the elution volume on the logarithm of the molecular weight can be used to calculate the molecular weight of particular protein peaks whose position is determined by analysis or absorbance measurements for inactive proteins, and by assays for enzymes. Hepatic cytosol preparations from Salmo gairdneri, Salmo trutta, Galaxias maculatus, G. truttaceus and G. auratus were examined by Sephadex gel column chromatography with a 1 mM GSH buffer, and assayed for activity toward C^{14}-TCIN and CDNB, the reference substrate for G S-transferase activity.

Electrophoresis is a well known technique for the separation of proteins into sharp bands by migration through a small pore size gel in an electric field whose final positions, dependent on molecular size, charge and polarizability, are characteristic in a particular buffer system. Changes in single amino acids within a protein produce a change in charge, and hence in electrophoretic mobility, in approximately 30% of proteins subject to such allelic differentiation. Electrophoresis is, therefore, a powerful tool in the study of phylogenetics when studying isozymes for which a characteristic stain exists, and which demonstrate detectable allelic differences in electrophoretic mobility (Fergusson, 1980). It was decided to investigate the salmonid and galaxiid TCIN detoxication enzymes by electrophoresis. Methods for developing a suitable stain for GST were also investigated.

8.2.2. MATERIALS AND METHODS

Gel column chromatography

Hepatic cytosol samples of all the species previously acclimated at least 1 week, with daily feeding to satiation with chopped earthworms, were prepared. Typically, 0.5-1.0 g liver in 5 vol. 0.01 M Tris-HCl (pH 8.3), 1 mM in GSH, was homogenized and centrifuged at 30,000 g for 1 h at 0-4° C. Supernatant cystosol was applied to a Sephadex G 75 gel column (30 x 2.6 cm) equilibrated with the above buffer. Fractions were eluted at 1.4 ml/min and collected each minute.

Combithek (Boehringer, GmbH) proteins were used for calibration, the relationship between log molecular weight and elution volume being linear.

0.10 ml of each fraction was assayed for activity towards C^{14}-TCIN as previously described (8.1.2), the aqueous assay fraction being radiocounted, and for activity toward CDNB, as described below. Protein analyses were performed by the method of Spector (1978). Absorbance of the fractions was measured at 280 nm.

CDNB assay for G S-transferase

The method of Habig et al. (1974) was used to assay for glutathione S-transferase by monitoring the rate of absorbance increase at 340 nm after addition of CDNB to the assay mixture, consisting of typically 2 ml of the above buffer and 0.10 ml or 0.20 ml gel eluant fraction. The basis for this assay is described in section 8.1.1.

Electrophoresis

Hepatic cytosol preparations and peak CDNB activity fractions from G. maculatus were prepared as above, mixed with 0.25 M sucrose and bromomethyl blue, applied to disc electrophoretic gel columns and run by the method of Davis (1964). 2% polyacrylamide stacking and 7.5% polyacrylamide separating gels were used, prepared by the method of Davis (1964). General protein staining of the freshly rimmed gels was performed using coomassie brilliant blue (1%) in 5% trichloroacetic acid solution.

Staining for GST activity was performed by the method of Clark (1982) with 0.04% starch incorporated into the gels. The staining method of Board (1980) was adapted, reversing the stain phases as follows. 0.1% starch was incorporated into the gels. Disc gel columns were sliced in half lengthwise and the cut faces were layed on filter paper soaked in 1% CDNB, 2% GSH in 0.1 M potassium phosphate solution (pH 6.5). These were then covered with "parafilm" and incubated for 40 min at 20, 25 or 30° C. The gels were then removed and the cut faces were placed onto special agar gels set in petri dishes for up to 12 h at room temperature. The agar gels had been prepared by mixing 2% molten agar solution with an equal volume of 0.9 ml 1% I_2 in KI diluted to 30 ml.

A further staining technique was attempted. Gels were prepared

as described by Davis (1964) with the HCl replaced with acetic acid. Gels were electrophoresed and immediately placed in a freshly prepared solution of 7 mM MeI, 6 mM GSH and 0.5% $AgNO_3$, in 0.1 M potassium phosphate, pH 7.0, and left for periods up to 2 h at room temperature with occasional gentle agitation. The same staining technique was also used on hepatic cytosol samples electrophoresed on "Cellogel".

Attempts were made to separate proteins by SDS electrophoresis of peak activity fractions from gel column runs of Salmo gairdneri hepatic cytosol. Protein bands, stained with the above general stain, were variable in position, and attempts were abandoned in the absence of purified enzyme preparations.

Freshly run disc gels were also radiostained for enzyme activity towards C^{14}-TCIN. Gels were washed in 0.1 M phosphate buffer (pH 7.5) and placed in a bath of 0.5 ug/ml C^{14}-TCIN (activity 6065 dpm/ug) and 1 mM GSH in 0.1 M Tris-HCl (pH 8.3). After incubation at room temperature for 30 min, gels were washed in buffer and sliced into 2 mm long discs. These discs were then extruded into 1.0 ml buffer through a hypodermic needle, and agitated for 2 h. The aqueous supernatant was extracted with 1 ml hexane, and 0.5 ml was radiocounted in Dimilume-30.

8.2.3 RESULTS

Molecular weights of highest enzyme activity toward C^{14}-TCIN and CDNB are shown in Table 8.2. The overlap of enzyme activities toward the two substrates was complete, as shown in Fig. 8.4 for S. gairdneri. However, in all cases peak activities occurred at slightly lower molecular weights for the C^{14}-TCIN substrate than for CDNB. Peak molecular weights all fell within the 30000 - 60000 range known for GST enyzmes (Jakoby, 1978). It appears, therefore, that hepatic GST activity toward TCIN is confirmed in all species tested.

Attempts to develop a stain specific for GST enzymes able to give the resolution required for good band position comparisons were unsuccessful. Typical band patterns for the MeI - GSH - H_2O_2 stain of Clark (1982) are shown in Fig. 8.5. These are similar to that illustrated in Clark's paper. Band definition is poor, with the bands being wide and diffuse. Varying the incubation times in the MeI GSH

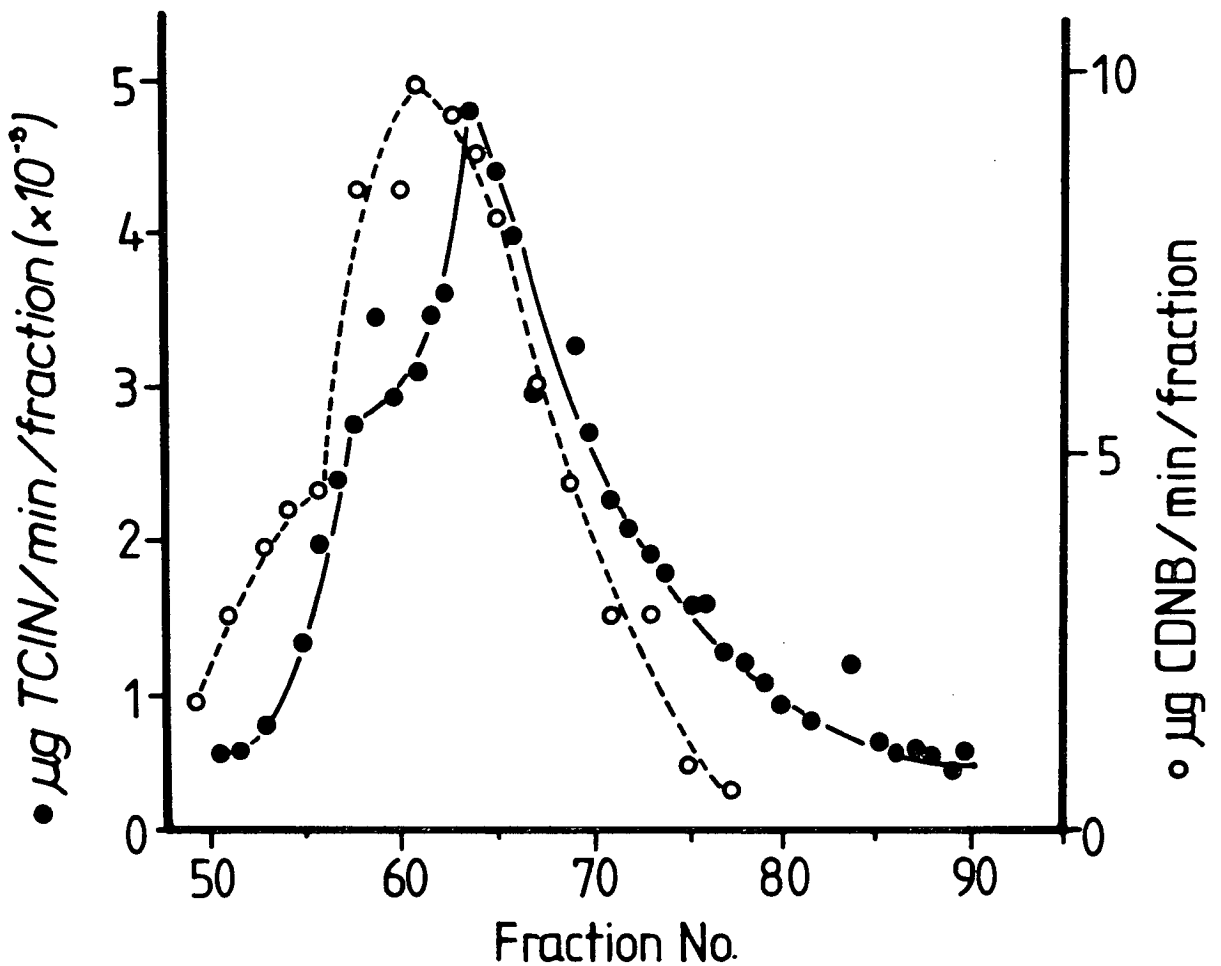


Fig. 8.4 Gel column chromatogram profile of S. gairdneri hepatic cytosol assayed for GST activity toward CDNB and C^{14}-TCIN. Origin of x-axis represents Vo; fraction size is 1.3 ml.

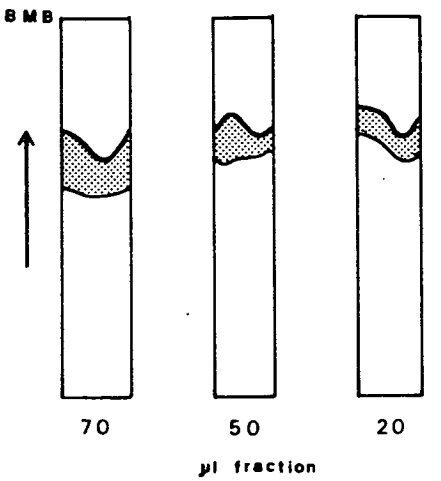


Fig. 8.5 Typical polyacrylamide electrophoretic stain patterns for G. maculatus peak GST gel column eluant fractions. BMB = dye front. Direction of travel is indicated.
Staining was with KI - GSH - H_2O_2.

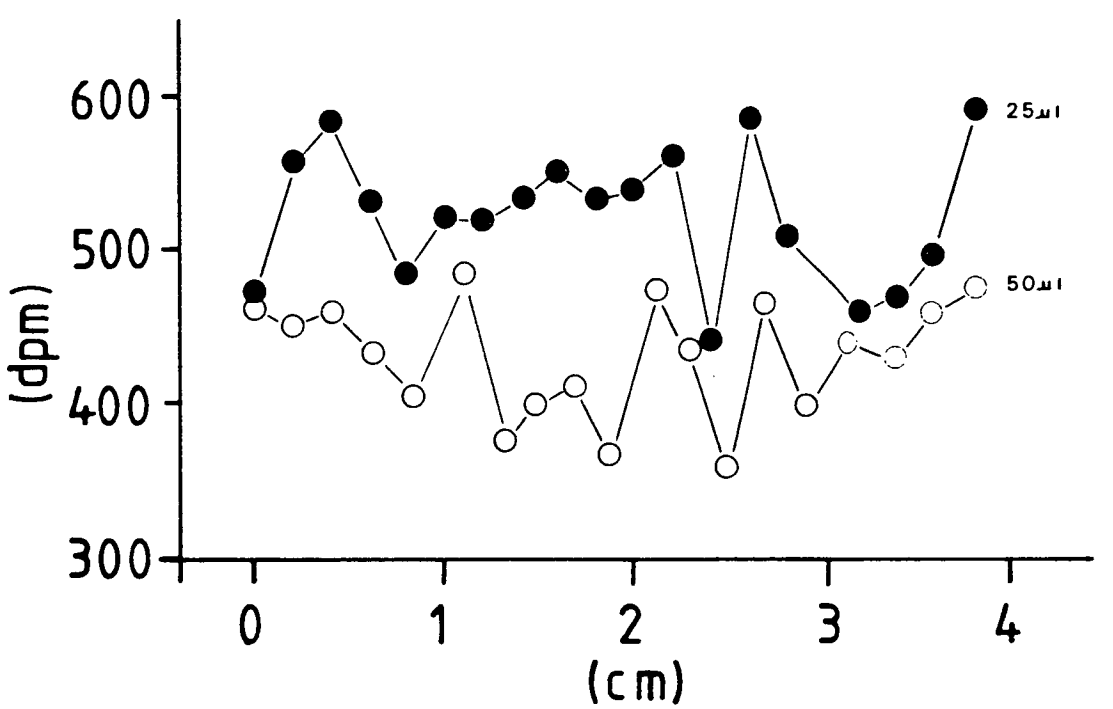


Fig. 8.6 Disc electrophoretic gel radiostain profiles for G. maculatus GST activity toward C^{14}-TCIN and GSH. Aqueous (conjugate product) radiolabel counts are shown for each gel section. Two volumes of gel column peak GST fraction were electrophoresed and stained. See text for details.

bath did not improve resolution, causing variations only in the intensity of the colour reaction. Protein bands of the same gels were, however, sharply defined.

The reverse staining adaption of the technique of Board (1980) gave the blue starch colour reaction, but with poorer definition than that occuring with the MeI - GSH - H_2O_2 stain. Again, varying conditions did not improve the quality of band sharpness.

The MeI - GSH - $AgNO_3$ stain caused precipitation within the gel of a general nature. Some initial opaqueness appeared in the areas of the previously stained bands, but the precipitation of a silver compound then proceeded throughout the gel rendering it entirely opaque.

Cellogel electrophoretograms gave a rapid precipitation of the expected yellow AgI at a particular location on the gel. However, the thinness and larger pore size of this type of gel is not applicable to the use of this precipitation reaction as a stain since the precipitate did not adhere tightly to the gel and band forms could not be defined.

Radiostaining gave some indication of multiple bands in disc gels but the amount of work and the expense involved was considered excessive for the variable results obtained (Fig. 8.6).

8.2.4 DISCUSSION

The work reported here represents the first study of the GST enzyme group in the Galaxiidae, and the first species-species comparison of GST in freshwater fish. Nimmo *et al.* (1979, 1981) described some basic properties of G S-transferases in *Salmo gairdneri* in comparison with the rat, showing the existence of at least three such enzymes of varying substrate specificity.

The GST group is known to demonstrate variability in activity toward substrates and the existence of multiple enzyme forms of GST is well known in higher vertebrates (Habig and Jakoby, 1981). Six isozymes occur in the rat (Jakoby *et al.*, 1976), five in humans (Habig *et al.*, 1976). In the squalids, five are known in the little skate, *Raja erinacea* (Foureman and Bend, 1979), and two in *Platyrhinoides triseriata* (Sugiyama *et al.*, 1981). Apart from the work of Nimmo and

colleagues, little is known of the GST enzymes in teleost fish. This study indicates that by substrate activity preferences, there are at least two GST enzymes in all five teleost species studied here. There also appears to be a consistent molecular weight difference between the GST enzymes of the galaxiids and the salmonids studied, the galaxiid GST being some 10000-20000 units lighter. More detailed chromatographic and electrophoretic analysis is needed to investigate the possible use of GST in phylogenetic studies.

The requirements for an electrophoretic enzyme stain are specificity, compatibility with the gel-buffer medium and visibility. A further requirement is that the stain remains localized to the area of enzyme activity. The MeI - GSH - H_2O_2 stain of Clark (1982) possesses all the former attributes but not the latter. Incubation of MeI and GSH gives I^- as the secondary product of the GST mediated conjugation reaction. Secondary incubation with acid hydrogen peroxide converts iodide to iodine providing a blue colour reaction with the starch incorporated into the gel. However, as soon as I^- is released it can freely diffuse through the gel matrix prior to fixation by oxidation and starch reaction. It appeared, therefore, that if the I^- can be reacted and localized within the gel as soon as it is produced, sharp band definition could be retained. The use of silver nitrate for this was attempted; the silver-iodide reaction results in rapid precipitation of bright yellow silver iodide. Although this gave definite "areas" of GST activity when attempted in "Cellogel", it was unsuccessful in polyacrylamide disc gels.

Although electrophoresis has not generally been used to study G S-transferase enzymes, probably due to the lack of a suitable stain, SDS electrophoresis has been used to investigate subunit molecular weight differences. It is a polyacrylamide electrophoretic technique in which the powerful detergent sodium dodecyl sulphate (SDS) is used to break up proteins into subunits. The fact that SDS electrophoresis also only shows separation based on molecular weight, and not charge, means that it is of limited use in the study of phylogenetics because only relatively major amino acid changes in proteins are sufficient to produce detectable changes in mobility. However, it has found utility in the study of G S-transferases, which are proteins composed of two subunits of roughly equal molecular weight, and where G S-transferase isozymes frequently show detectable differences in subunit mobility

(Habig et al., 1976). It is possible that SDS electrophoresis may show differences in subunit composition of phylogenetic interest between the GST enzymes of the Salmonidae and Galaxiidae, especially in the light of apparent molecular weight differences of the GST whole enzyme from the two groups.

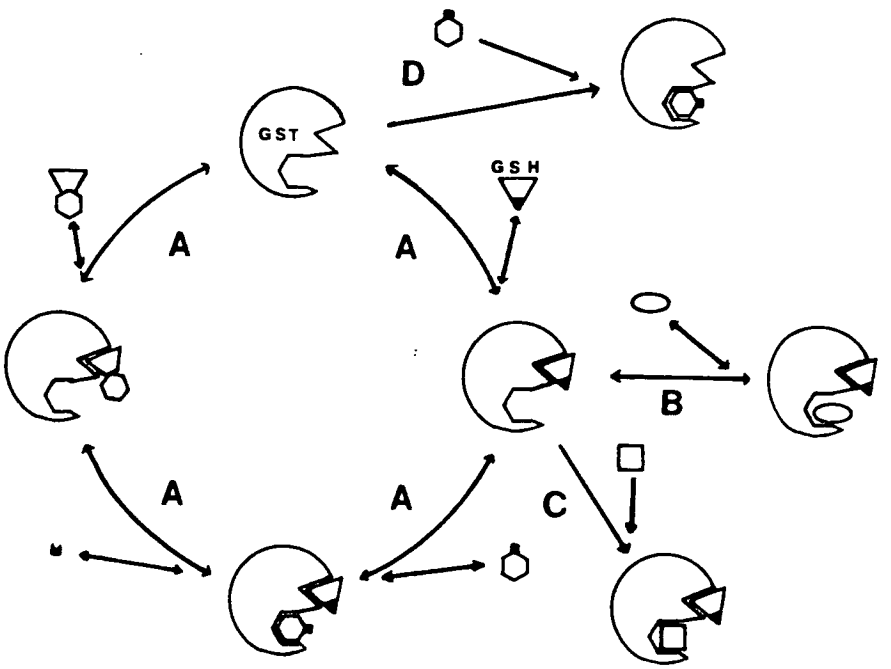
8.3 INDUCTION, BINDING BEHAVIOUR AND INHIBITION OF GLUTATHIONE S-TRANSFERASES

8.3.1 INTRODUCTION

The ability of a detoxication enzyme system to promote detoxication and excretion of xenobiotics is influenced not only by the nature of the electrophile and the concentrations of substrates but also by the available effective enzyme and the ability of the organism to induce it. Induction in phase I detoxication enzymes on exposure to phase I metabolizable xenobiotics is well established in fishes (Chambers and Yarborough, 1976; Bend et al., 1979). Phase II induction is well established in mammals. Xenobiotic exposure induction of epoxide hydrase, UDP-glucuronyl transferase and glutathione S-transferase has been demonstrated in rats (Bresnick et al., 1977; Bock et al., 1973; Kaplowitz et al., 1975; Baars et al., 1978). This section reports an investigation into induction of GST activity toward TCIN by TCIN pre-exposure, in Salmo gairdneri, Galaxias maculatus, and G. truttaceus.

The G S-transferases are known to show a high non-covalent binding ability with endogenous bile pigments, where they play a major role in bilirubin transport in the liver, with estrogens, fatty acids, hemes and gastrin and with exogenous compounds such as BSP, rose bengal, penicillin and ethacrynic acid (Arias et al., 1976).

Jakoby (1976) proposed the following scheme by which GST enzymes can become involved in detoxication processes, by three modes:



where A = catalytic sequence for GSH conjugation with a reactive nucleophile

B = reversible binding with a non reactive but bindable substrate

C = irreversible binding with a substrate reactive enough to covalently bind the enzyme in the presence of GSH.

A further mode of detoxication can also be proposed. Irreversible binding of a reactive substrate to GST could occur when GSH levels are depleted by conjugation reactions with the electrophile. This is particularly important with regard to maintaining detoxication efficiency, when exposure to an electrophile depletes GSH reserves. This mode of detoxication, one involving enzyme "suicide", is represented by the sequence D in the above diagram.

This section describes an investigation into the induction of GST on TCIN pre-exposure. It also describes experiments conducted to investigate the TCIN - binding properties and the inhibition of Galaxias maculatus GST, and to determine some kinetic parameters for

the TCIN - GSH and CDNB - GSH reactions in the presence of G. maculatus GST. The effect of acephate, the organophosphate insecticide aerially sprayed with TCIN (see section 2.1), on GST activity toward TCIN and GSH is also reported.

8.3.2 MATERIALS AND METHODS

Induction of GST activity toward TCIN

Four individuals each of S. gairdneri, G. maculatus and G. truttaceus were exposed to TCIN at each of 0, 2.2, 4.4 and 9 ug/l for four days in the flow-through toxicant delivery system. Details of exposure protocols, concentration, monitoring and analysis are as in Chapter 3. Fish were killed (tricaine methanosulphonate) and liver cytosol preparations assayed for activity toward C^{14}-TCIN and GSH as described in 8.2.2. Protein pellets from the assays were prepared and analysed as in 8.2.2.

C^{14}-TCIN binding to GST in G. maculatus

High GST activity fractions from the gel column separations described in 8.2.2 were passed through the same column with buffer A in the absence of GSH. 200 ul peak fraction of this second column run were reacted with C^{14}-TCIN, both with and without GSH in 0.1 M Tris - HCl (pH 8.3) buffer for 20 min, each in triplicate. Half of each reaction mixture was stopped with TCAA and worked up as in 8.2.2. The remaining halves were reacted with C^{14}-TCIN in above buffer with 1 mM GSH for 2 min and worked up as in 8.2.2.

Reaction characteristics

Assays were performed with G. maculatus cytosol for activity toward C^{14}-TCIN at a series of TCIN concentrations up to 0.5 ug/ml, with 1 mM GSH concentration in 0.1 M Tris-HCl (pH 8.3) buffer. Hexane and water fractions from the assay extraction mixtures were radiocounted and used to give measures of TCIN utilization and glutathione conjugate production rates.

Assays were also performed, using CDNB as substrate, on the same cytosol by the method of Habig et al. (1974) at CDNB concentrations up to 54 ug/ml.

Effects of acephate on activity toward C^{14}-TCIN

C^{14}-TCIN assays were performed with peak enzyme C^{14}-TCIN activity fractions from the gel column separation of G. maculatus cytosol (section 8.2.2). Assays, performed in triplicate, were performed using buffer A made up with Orthene (R) (ICI) to give final assay concentrations of 20 and 100 ug/l, and 20 and 50 mg/l acephate. Incubation was for 10 min using 100 ul eluant fractions in 2.0 ml acephate - buffer A solution, and work-up as in 8.2.2.

8.3.3 RESULTS

Significant induction of GST activity toward C^{14}-TCIN was shown in hepatic cytosol of S. gairdneri, G. maculatus and G. truttaceus on pre-exposure to TCIN at low concentrations for 4 days (Fig. 8.7). The inductive response appears to be concentration dependent. Protein binding in the hepatic cytosol of G. maculatus was decreased markedly on pre-exposure to TCIN, and also appears to have decreased slightly with time of assay incubation, consistent with the in vitro binding behaviour observed in Chapter 7 (Fig. 8.8).

Several trends were revealed by the binding study (Fig. 8.9). There was a marked decrease ($p < 0.01$) in aqueous C^{14}-TCIN metabolite production by hepatic cytosol when incubated in the absence of GSH in the buffer (A, Fig. 8.9). This confirms that a GST catalysed, GSH dependent reaction is the primary mode of TCIN metabolism. Residual activity may be due to GST-bound GSH (Kosower and Kosower, 1976). The protein bound level of C^{14}-TCIN was increased substantially during incubation with cytosol without GSH in the buffer.

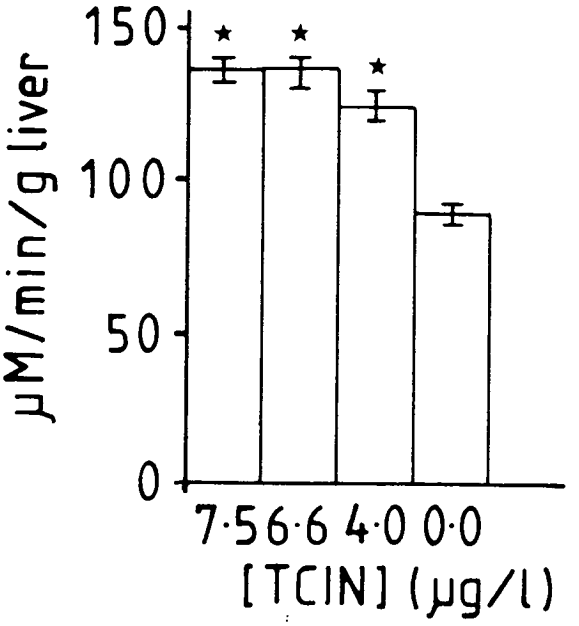
The subsequent degree of conjugation of C^{14}-TCIN with GSH was markedly decreased ($p < 0.0001$) when compared to that when the cytosol had been previously incubated with C^{14}-TCIN with GSH in the buffer. These results indicate that C^{14}-TCIN can bind the hepatic GST when GSH concentrations are low. The binding is irreversible, probably covalent, and inhibits the enzyme activity. GSH, therefore, appears to play a protective role in the prevention of TCIN-protein binding, at least in the case of GST.

Direct linear plots of rate (V) aginst substrate concentration were made in order to estimate the Km and V values for TCIN and

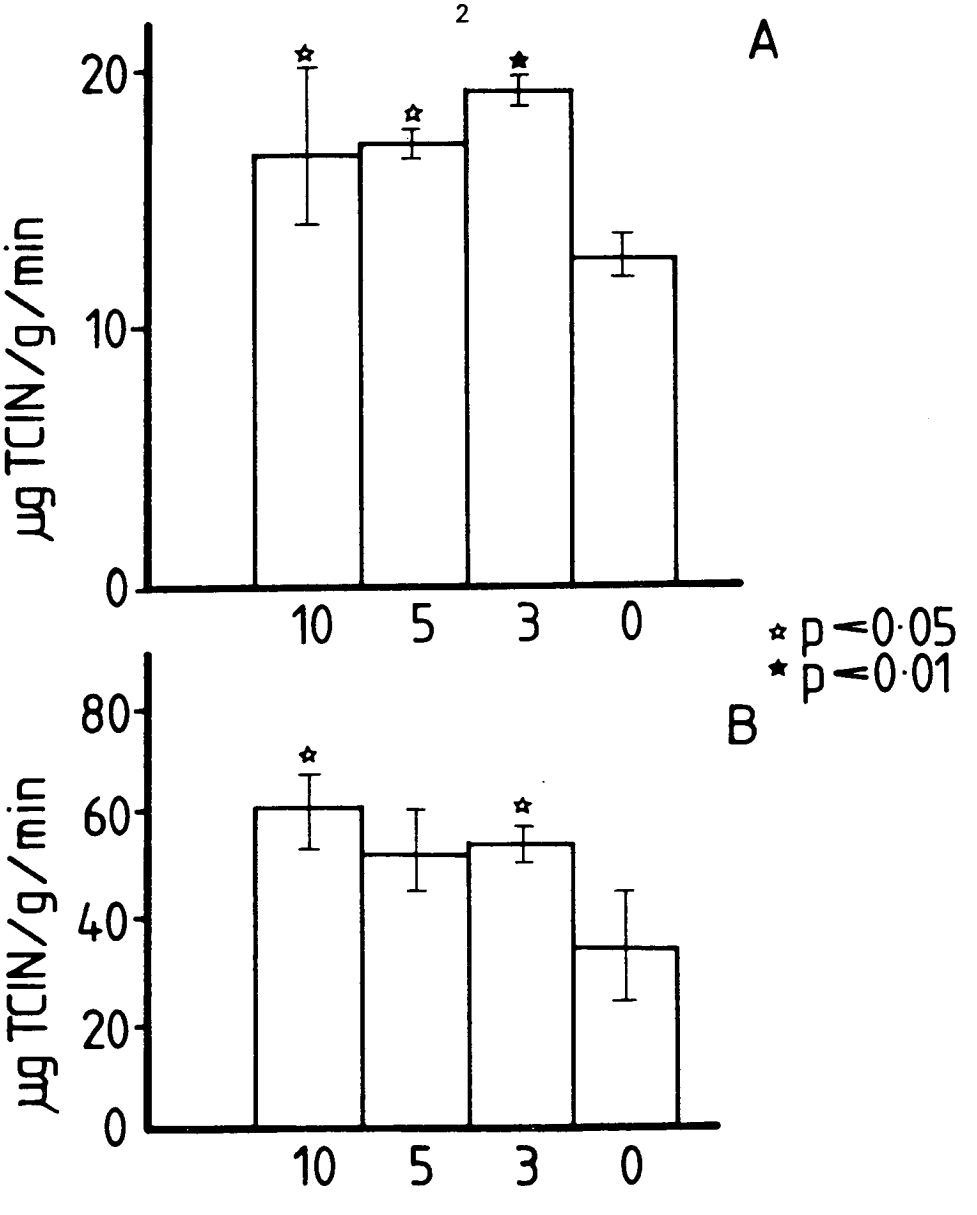
Fig. 8.7 1. Induction of GST activity toward C^{14}-TCIN with 96 h exposure to TCIN in S. gairdneri. (*) = $p < 0.001$.

2. Induction as above in G. maculatus (A), and G. truttaceus (B), after exposure to TCIN for 96 h and 110 h respectively.

1


150
100
50
0
μM/min/g liver
7·5 6·6 4·0 0·0
[TCIN] (μg/l)

2


A
20
10
0
μg TCIN/g/min
10 5 3 0
☆ p<0·05
★ p<0·01
B
80
60
40
20
0
μg TCIN/g/min
10 5 3 0

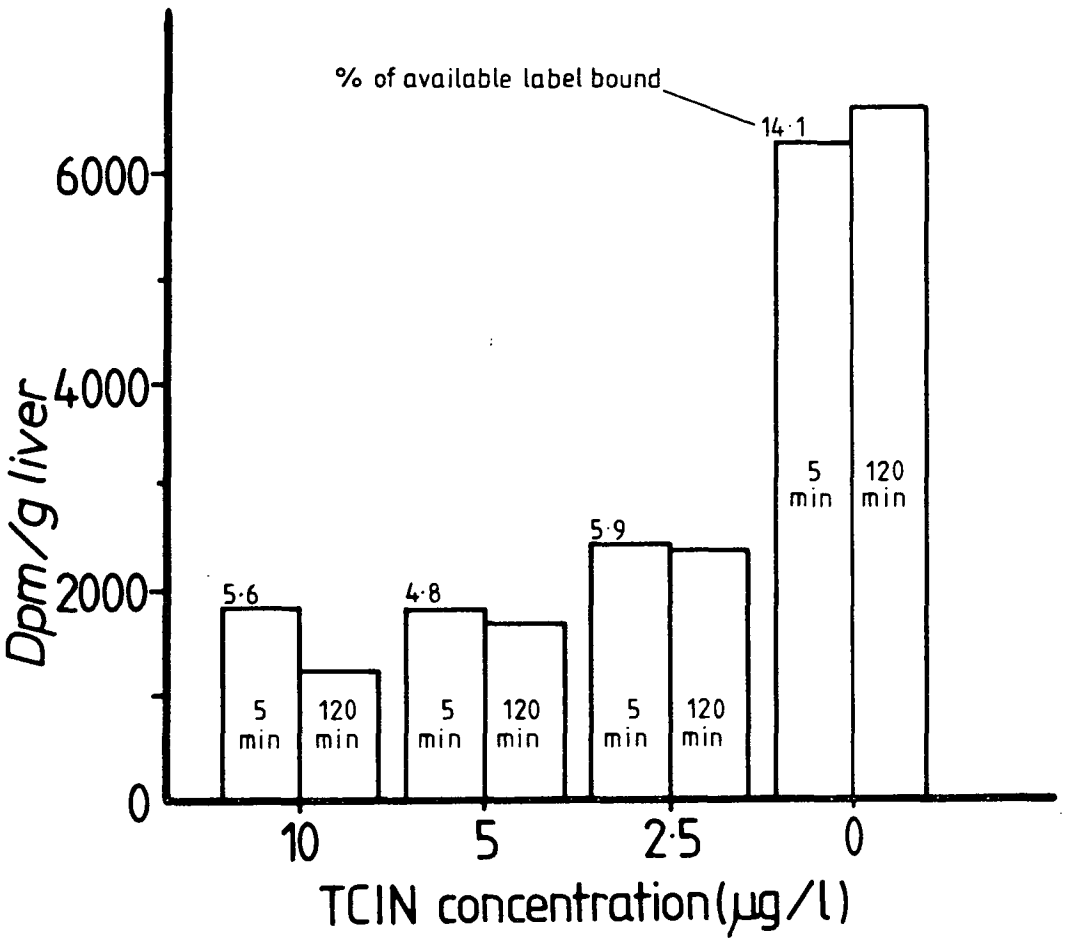


Fig. 8.8 Protein bound C^{14}-TCIN after 5 and 120 min incubation with standard 1 mM GSH assay mixtures of G. maculatus hepatic cytosol from fish previously exposed for four days to varying levels of TCIN.

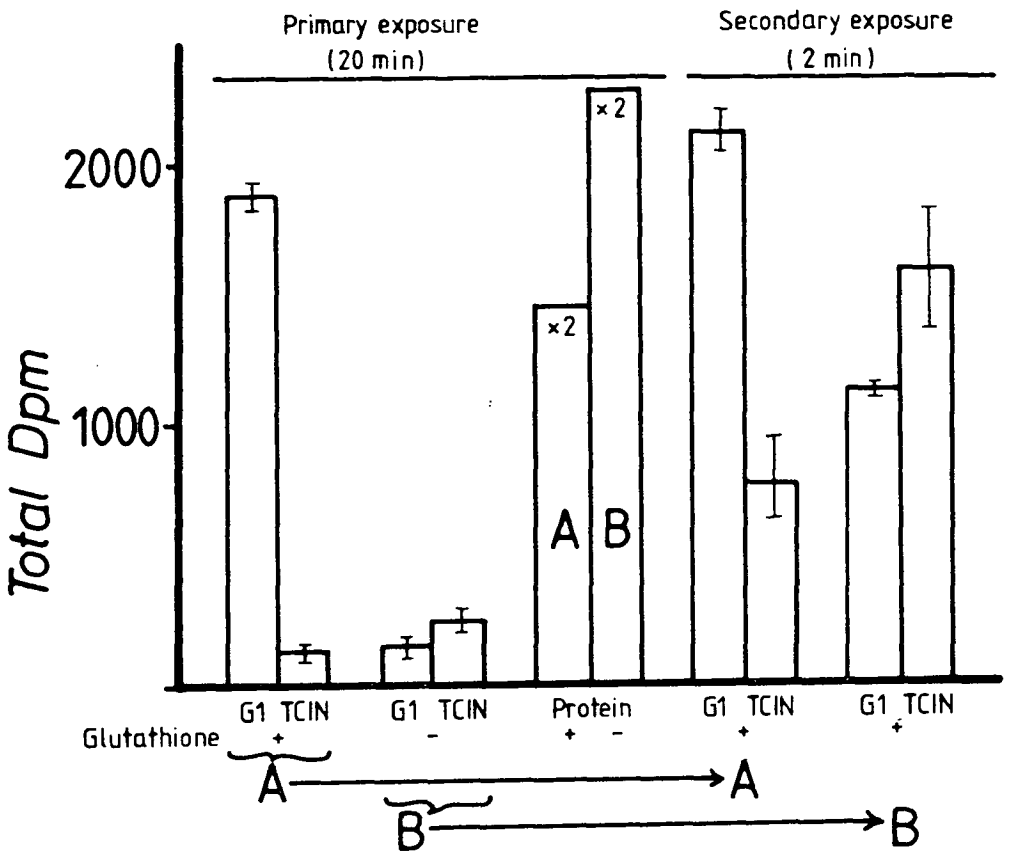


Fig. 8.9 Binding-induction experiment.

C^{14}-TCIN, glutathione conjugate (G1) and protein-bound residue levels from assay mixtures of G. maculatus hepatic cytosol, of peak GST activity, without GSH, exposed to C^{14}-TCIN for 20 min and then after 50% dilution, for 2 min.
A = mixtures exposed with 1 mM GSH in the buffer,
B = mixtures exposed without GSH.

CDNB in GST catalyzed conjugation with GSH, by the method of Cornish-Bowden (1979). Values of Km and V estimated from the above as well as from Linneweaver-Burke plots are shown in Table 8.3. The values obtained from both plotting methods agree well, although direct linear plots gave lower error values.

Acephate did not inhibit GST activity toward C^{14}-TCIN up to a concentration of 50 mg/l (Table 8.4).

8.3.4 DISCUSSION

This work is the first established case of G S-transferase induction in fish by a phase II metabolizable toxicant. It is interesting to note that the C^{14}-TCIN protein-binding ability of hepatic cytosol preparations decreases with pre-exposure to TCIN. It is possible that an increase in GSH conjugation efficiency causes a decrease in the availability of C^{14}-TCIN for binding in the assays. The result could also indicate that binding to available sites has already occurred during the pre-exposure period. In any event, if TCIN toxicity is due to protein binding, pre-exposure to low levels of TCIN may markedly influence the subsequent binding efficiency and possible toxic action of TCIN, in combination with an inductive effect on conjugative activity toward TCIN.

Since acephate is often sprayed as a mixture with chlorothalonil (2.1.1), it is likely, in those areas sprayed, that fish are exposed to both chemicals from run-off. Acephate is of very low toxicity to fish. The 24 h LC50 to Salmo gairdneri is 2890 mg/l (Klaverkamp, 1982). Inhibition of GST by acephate is unlikely on chemical considerations and known metabolic pathways for organophosphate compounds (Miyamoto et al., 1979).

Pabst et al. (1974) demonstrated the irreversible covalent binding of CDNB to rat G S-transferase A. This ability to bind substrates irreversibly, promoted by the absence of GSH, is demonstrated for Salmo and Galaxias G S-transferases with C^{14}-TCIN. The inactivation of the enzyme after binding is consistent with the covalent binding being associated with the active substrate binding site, and the role of a thiol group is implicated.

Rat glutathione S-transferase A has been shown to have two

Table 8.3 Reaction characteristics of the conjugation of C^{14}-TCIN and CDNB with glutathione by G. maculatus hepatic G S-transferase

Substrate	Reaction order (r^2) Species	Km umol (SD)	V umol/min (SD)	Substrate conc. ug/ml	GSH mM
C^{14}-TCIN	0.968 C^{14}-TCIN (0.995)	5.40 (1.20)	0.396 (0.074)	0.4-0.6	1.0
	1.033 conjugate (0.981)			0.4-0.6	1.0
CDNB	0.888 conjugate (0.974)	1.35×10^{4} (0.59×10^{4})	1.602×10^{-3} (0.356×10^{-3})	100	1.0

Table 8.4 Effect of acephate on G S-transferase activity toward C^{14}-TCIN

Acephate concentration	Activity ug/g/min (SD)
0.0	6.20 (0.59)
20 ug/l	5.68 (1.84)
100 ug/l	5.55 (1.074)
20 mg/l	6.21 (0.81)
50 mg/l	6.79 (1.03)

reaction mechanisms depending on whether GSH concentrations are lower or higher than 0.15 mM (Pabst et al., 1974). At high (>0.15 mM) GSH concentrations the enzyme was found to proceed by an ordered sequential mechanism in which glutathione binds first to the enzyme and then to the electrophilic substrate. The G S-conjugate of CDNB was found to be competitive with GSH and non-competitive with CDNB. GSH was found to give normal saturation behaviour above 0.1 mM with an apparent Km of 0.2 mM.

Log-log plots of reaction rates for TCIN depletion and G1 production with G. maculatus GST showed that the reaction was first order for reactant and product when the GSH concentration was constant. It also appears that CDNB product appearance rates are first order with respect to CDNB concentration, although the agreement is not as good.

When comparing the reaction rates of two possible substrates for an enzyme, the important factor in determining the "favoured" substrate is V/Km (Cornish-Bowden, 1979). Consequently, when comparing any two reactions with substrates, A and B respectively, catalyzed by the same enzyme, the rate ratio is given by:

$$\frac{V_1}{V_2} = \frac{(V^A/K^Am).a}{(V^B/K^Bm).b} \qquad \text{Eqn. 8.1}$$

On the basis of this equation, G. maculatus GST is found to catalyze the conjugation of TCIN with GSH more efficiently than that of CDNB with GSH by a factor of 6.2×10^3, when substrate concentrations are the same (a = b).

There are two possible reasons for this enhanced catalytic ability towards TCIN. Firstly, the electrophilic reactivity of TCIN with GSH may be higher, and, secondly, the GST may be activating TCIN more favourably over CDNB. The first possibility may be examined by comparing the uncatalyzed reaction rates of TCIN and CDNB with GSH. Typical values are shown in Table 8.5. Since GSH is in great excess, and since $-(da/dt) = k.a_o^n$ and n, the reaction order, is unity, it is possible to compare directly these reaction rates after adjusting to the same substrate concentration. When this is done, it is found that the

Table 8.5 Typical uncatalysed reaction rates of CDNB and C^{14}-TCIN with GSH under standard assay conditions

Substrate	Concentration (uM)	Rate (uM/min)
CDNB	153	1.105×10^{-3}
C^{14}-TCIN	2.43	4.029×10^{-4}

ratio of reaction rates is given by:

$$\frac{-d(TCIN)}{dt} \Big/ \frac{-d(CDNB)}{dt} = \frac{k^1(TCIN)}{k^2(CDNB)} = 23.0$$

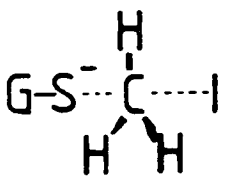
Hence, the reactivity of TCIN with GSH is higher than CDNB, probably by virtue of its higher electronegativity due to multiple chlorine substitution. The catalyzed reaction of TCIN with GSH is still higher than that of CDNB, however, by a factor of 300. This may be due to some form of preferential activation in the enzyme.

Jakoby (1982) discussed the question of whether the natural substrates of detoxication enzymes are xenobiotic compounds. He suggested that, despite low catalytic efficiency, the low specificity of these enzymes allows them to be of great utility in removing lipophilic foreign compounds, "the garbage of the cell", and that they are of vital importance in "disposing of garbage of such uncatalogable diversity". The GST enzymes appear to have sacrificed catalytic efficiency for diversity of "scavengeable" substrates, and in fact have been called "a very weak kind of enzyme - an enzyme in quotes" (Jakoby et al., 1976). Rate increases in the presence of enzyme are of the order of 100 times the uncatalyzed reaction, compared with rate increases at least 10^5 times in metabolic enzymes of high specificity. Jakoby regards the GST enzymes as a binding protein which bind a number of electrophiles passively with low specificitiy. It also has the ability to bind GSH and to activate it to the thiolate anion, GS^-. Its ability to catalyze the formation of GS^- may be as effective as any enzyme. However, its apparent catalytic ability depends on the reactivity of the secondary electrophilic substrate which is not activated, but bound passively. Some "activation" of the electrophile may occur by a proximity effect.

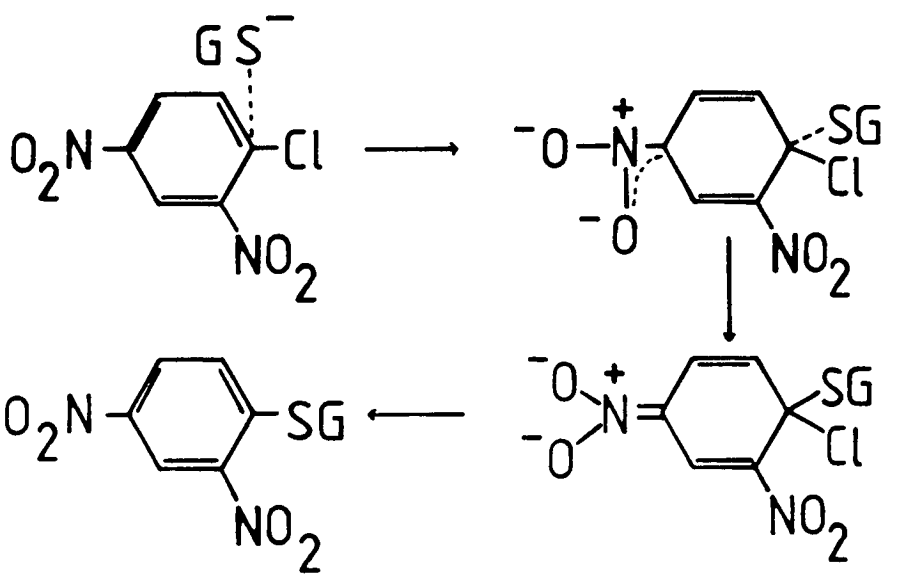
Evidence for this passive interaction with substrates comes firstly from the large number of substrates that are bindable. It is very unlikely that the GST enzyme would be able to bind every substrate, so that its steric arrangement is optimally favourable for the conjugation reaction, and that every one of the great diversity of catalyzable electrophilic functions is "activated" in some way.

Secondly, it is found that the GSH conjugation with alkanes such as MeI must proceed by classic SN1 displacement reactions, whereas only an addition-elimination reaction sequence is envisaged for aryl substitutions such as in CDNB as shown here (from Jakoby et al., 1976).

Displacement (MeI):



Addition - replacement (CDNB):



These two mechanisms proceed by two distinct routes. In the case of MeI, the GS^- ion attacks from the "rear" of the molecule, whereas with CDNB, the GS^- ion attacks from above the plane of the ring. Given that a given G S-transferase can catalyze both of these conjugations, it cannot be performing any specific activation of the substrates. Indeed, high specificity is regarded as detrimental to an enzyme of such broad substrate requirements.

Consequently, the possibility of a specific "activation" of TCIN by GST is highly unlikely. There is, however, one way in which TCIN may show a distinct reactive advantage over CDNB when found in the enzyme binding site. CDNB has only one reactive site, whereas TCIN has up to three. Given that binding is not stereo-specific for such

aromatic nuclei (witness the presence of high biliary levels of the di-conjugate, Chapter 7), then the likelihood of TCIN having a reactive site proximal to the activated GSH is much higher than for CDNB. Consequently, a higher "catalyzed" reaction rate may be expected for TCIN. A secondary effect may be the higher affinity of TCIN for binding to organic residues than CDNB. This may enhance the binding rate to GST and hence the reaction rate of TCIN with GSH.

The G S-transferases are widely distributed in the animal kingdom. They are known to occur in humans (Baars et al., 1981), rats (Jakoby et al., 1976; Scully and Mantle, 1981), crustaceans (Tate and Herf, 1978), earthworms (Stenersen and Øien, 1981), insects (Motoyama and Dautermann, 1978, 1979) and various species of marine fish and squaliids (James et al., 1976). Nimmo et al. (1979, 1981) and Bauermeister et al. (1983) studied the properties and distribution of GST, GSH and γ-glutamyl transpeptidase in Salmo gairdneri and concluded that a mercapturate detoxication pathway exists in that species. The work described here confirms those findings with regard to TCIN metabolism, extending the known range of GST occurrence to include Salmo trutta and three species of the Galaxiidae. The role of GST in TCIN metabolism is established, but a study of the relationship between GST, its induction, GSH and thiol-rich enzymes is necessary in order to delineate the role of GST in TCIN detoxication. This is the subject of Chapter 9.

CHAPTER 9

EFFECTS OF TCIN ON ENZYME ACTIVITIES AND THIOL LEVELS

9.1.1 INTRODUCTION

That foreign organic compounds have important effects on enzyme activities is a well established fact in mammals (La Du et al., 1972), and has been demonstrated in fish (Khan et al., 1979). Two primary interactions occur: induction and inhibition. Both processes are known to occur in fish, though detailed work has been performed in only a few areas.

Bend et al. (1979) demonstrated induction of mixed-function oxidase microsomal activity on pre-exposure of the little skate Raja erinacea, to polycyclic aromatic hydrocarbons. Similarly, the same enzyme system was shown to be inducible in Salmo gairdneri on pre-exposure to a variety of aromatic hydrocarbons (Elcombe et al., 1979; Egaas and Varanasi, 1982). Levels of cytochrome P450 and monoxygenase activity have been found to be induced in sheepshead minnow, Archosargus probatocephalus and the common carp, Cyprinus carpio, on pre-treatment with PCB's and aryl hydrocarbons respectively (James and Weatherby, 1978; Melancon et al., 1981). Environmental levels of organochlorine pesticides were found to induce the cytochrome P450 system in C. carpio, Tilapia and S. gairdneri (Yahalomi and Perry, 1981).

With regard to phase II detoxication enzymes, it is of interest to note that PCB exposure did not affect glutathione S-transferase activity in sheepshead minnow (James and Weatherby, 1981). Sastry and Sharma (1979) found that endrin, an organochlorine pesticide, induced activity in three phosphatase enzymes in Ophiocephalus punctatus, indicating possible effects on gluc-ogenesis. In contrast, Addison and Zinck (1977) found that pre-administration of DDT and derivatives to brook trout (Salvelinus fontinalis) had no effect on the subsequent rate of dehydrochlorination of DDT. In general, however, it appears that the number of fish species reported demonstrating the

response of induction of detoxifying and associated metabolic enzymes is increasing (Pickering et al., 1983).

Inhibition of enzymes is often associated with the onset of toxic symptoms. Brain acetylcholinesterase inhibition is a well established toxic response in fish (Coppage, 1972; Klaverkamp and Hobden, 1980) and has been used for diagnosis of fish exposure to organophosphate insecticides such as acephate (Rabeni and Stanley, 1979). Oxygen analogs of several organophosphates have been shown to be potent inhibitors of fish muscle esterases (Whitmore and Hodges, 1978). The well known oxidase inhibitor, piperonyl butoxide, was also shown to inhibit the activity of the mixed-function oxygenase system of rainbow-trout (Melancon, 1979).

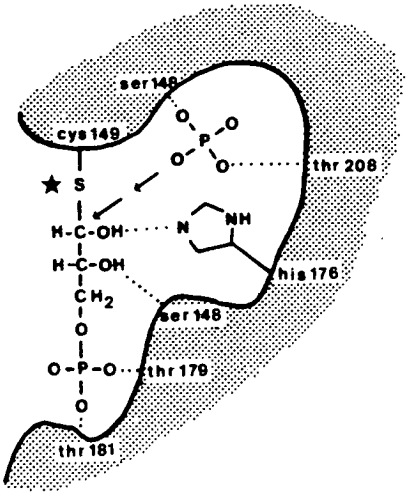
Apart from considerations of enzyme behaviour on exposure to xenobiotic organics, investigation of effects on the concentrations of secondary substrates necessary for conjugative detoxication are also vital. Little or no such work has been performed in fish toxicological studies. Particularly relevant in this study is the relationship between TCIN exposure and glutathione concentrations, especially in the liver.

Depletion of glutathione (GSH) by electrophiles in the rat and mouse has been studied extensively, and was reviewed by Chasseaud (1976), who reported decreases of up to 90% hepatic GSH, depending on time and strength of dose and on the type of administered electrophile. Kidney GSH levels are also depleted in mice when administered with p-aminophenol (Grove et al., 1979). In a similar vein, Siegel (1970 a,b) demonstrated that the trichloromethyl sulfenyl fungicides caused extensive depletion of GSH in fungal cells. Buckley (1981, 1982) has shown that administration of monochloroamine, a powerful oxidative toxicant, caused severe depletion of GSH in erythrocytes of Salmo gairdneri in vitro, and he also demonstrated a lack of complete protection by GSH against the oxidation of haemoglobin in the blood cells. GSH levels could be restored in erythrocytes when administered with glucose and mercaptoethanol (Buckley, 1981).

It is known that acetaminophen causes GSH depletion in mice consequent with increased lipid peroxidation (Thelen and Wendel, 1983) and cellular damage in the liver. Parker et al. (1981) studied the metabolism of acetaminophen by isolated S. gairdneri hepatocytes. Significant quantities of glutathione conjugates were produced. It is likely, therefore, that acetaminophen also causes GSH depletion in S. gairdneri on exposure; this may also apply to TCIN in the same

species.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an enzyme which catalyzes reversibly the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate. It is a key enzyme in the glycolytic conversion of glucose to pyruvate - a principal pathway in carbohydrate metabolism in most organisms. It contains four catalytic subunits each with an active site containing a cysteinyl thiol group which binds the substrate as a thioester. There are up to four cysteinyl residues per subunit but Cys-149 is the most reactive, and is essential in the catalytic process. Partial or complete inhibition of the enzyme by acetylating agents has been related directly with blocking of the Cys-149 thiol residue (Stallcup and Koshland, 1973 a,b) in one or all the subunits. The active site of GAPDH is shown here:



Active site of GAPDH (Redrawn from Tamm, 1982). The reactive thiol residue is indicated.

TCIN has been shown to be an inhibitor of the specific NAD thiol-dependent glycolytic and respiratory enzymes GAPDH, alcohol dehydrogenase and malate dehydrogenase, both in vitro and in cells of the fungus Saccharomyces pastorianus (Tillmann et al., 1973). Significant inhibition in fungal cells only occurred when the reaction between TCIN and GSH had caused most or all of the GSH reserve to be depleted. Cell mortality was directly correlated with the loss of thiol-enzyme activity and not with the formation of GSH-TCIN derivatives. Long and Siegel (1975) studied the reaction of GAPDH and αCT with TCIN in vitro. TCIN did not react with the non-thiol protein αCT, but reacted with GAPDH causing inhibition when in the absence

of low-molecular weight thiol groups. It appeared that TCIN reacted with the Cys-149 thiol residues only, by irreversible covalent binding.

This chapter describes an investigation into the effect of TCIN exposure at lethal and sublethal levels on the concentration of hepatic low-molecular weight thiols, and the activities of GST and GAPDH. These experiments were performed in order to assess whether GAPDH is inhibited by TCIN *in vivo* and whether this effect is a major contributor to TCIN toxicosis in fish, as it is in fungi, as well as to examine the concomit-ant extent of GSH depletion in the liver and the possible role of GST induction on sublethal exposure to TCIN. A further investigation into GAPDH inhibition by TCIN *in vitro* is also presented.

9.1.2 MATERIALS AND METHODS

Salmo gairdneri (9–12 g) were supplied by Sevrup Fisheries Pty. Ltd., Bridport, Tasmania. *Galaxias truttaceus* were collected by electrofishing. Acclimation and feeding protocols and the flow-through apparatus for exposure experiments were as described previously (3.1.2). Exposures at various concentrations of TCIN ranging from 2 to 30 ug/l were carried out as described previously (3.1.2).

Groups of five *Galaxias truttaceus* were exposed to 0, 3, 6 and 8 ug/l TCIN for 115 h, and to 13 ug/l for 50 h without feeding. *Salmo gairdneri* were exposed to 0, 10 and 30 ug/l with feeding. Four fish were taken from each treatment at intervals from 0 to 16 h. All fish were killed (tricaine methanosulphonate) and dissected immediately.

Liver homogenates were prepared as described previously (8.1.2), using 8 vol. 0.1 M Tris-HCl(pH 8.3) buffer.

Total soluble thiol, GSH and GST assays were carried out by the methods of Ellmann (1956), Habig *et al.* (1974), and as described in 8.1.2. GAPDH activity was determined by the method detailed in Long and Siegel (1975), with the forward reaction being monitored at 340 nm. Inhibition of GAPDH by TCIN was studied as outlined by Long and Siegel (1975) using GADPH standards in 0.1 M Tris-HCl(pH 8.3) 0.05 mM in DTT, at 0.017 nmole/ml GADPH. Inhibition of GADPH in cytosol preparations was studied the same way at two assay GAPDH

concentrations (0.516 and 0.017 nmole/ml). Some TCIN - GAPDH incubation mixtures were made 1 mM in GSH. All assays were performed in triplicate.

GADPH was obtained from Boehringer GmbH as a suspension and used as a standard for cytosol GADPH activity measurements. NAD, GSH and Glycerinaldehyde-3-phosphate diethylacetal were obtained from Boehringer GmbH. CDNB was A.R. grade; TCIN was purified technical grade material. All other chemicals were A.R. grade.

9.1.3 RESULTS

Hepatic thiol levels in unfed G. truttaceus exposed to a range of TCIN concentrations were significantly reduced with respect to controls (Fig. 9.1).

Liver GSH levels in fed Salmo gairdneri exposed to 30 ug/l TCIN were not significantly different from controls (Fig. 9.2). Fish exposed to 10 ug/l showed increases in liver GSH after 48 h (Fig. 9.3). Cytosol thiol levels were all higher than GSH levels. They showed no significant difference from controls except at 84 h of 10 ug/l TCIN exposure.

GST activity, assayed with CDNB, showed marked induction on exposure to 10 ug/l (Fig. 9.4). This confirms the induction of GST observed in Chapter 8 on TCIN exposure for 96 h. However, exposure to 30 ug/l caused no difference from controls, although some decrease in activity is observed at or near lethal exposure times.

Liver GAPDH activity in S. gairdneri was not significantly affected by TCIN exposure. A transient decrease in activity occurred on exposure to 30 ug/l (Fig. 9.5).

Cytosol preparations of S. gairdneri liver showed no decrease in GAPDH activity at an assay concentration of 0.516 nmole/ml GAPDH when exposed to concentrations of 0.3, 0.6, 1.2 and 1.8 ug/l TCIN for 2.5 h (Fig. 9.6). Significant decreases in cytosol GAPDH activity did occur, however, at all concentrations where the assay GADPH concentration was 0.017 nmole/ml. The degree of inhibition increased curvilinearly with TCIN concentration, demonstrating the existence of a limiting factor.

The degree of inhibition of pure GAPDH preparations by TCIN

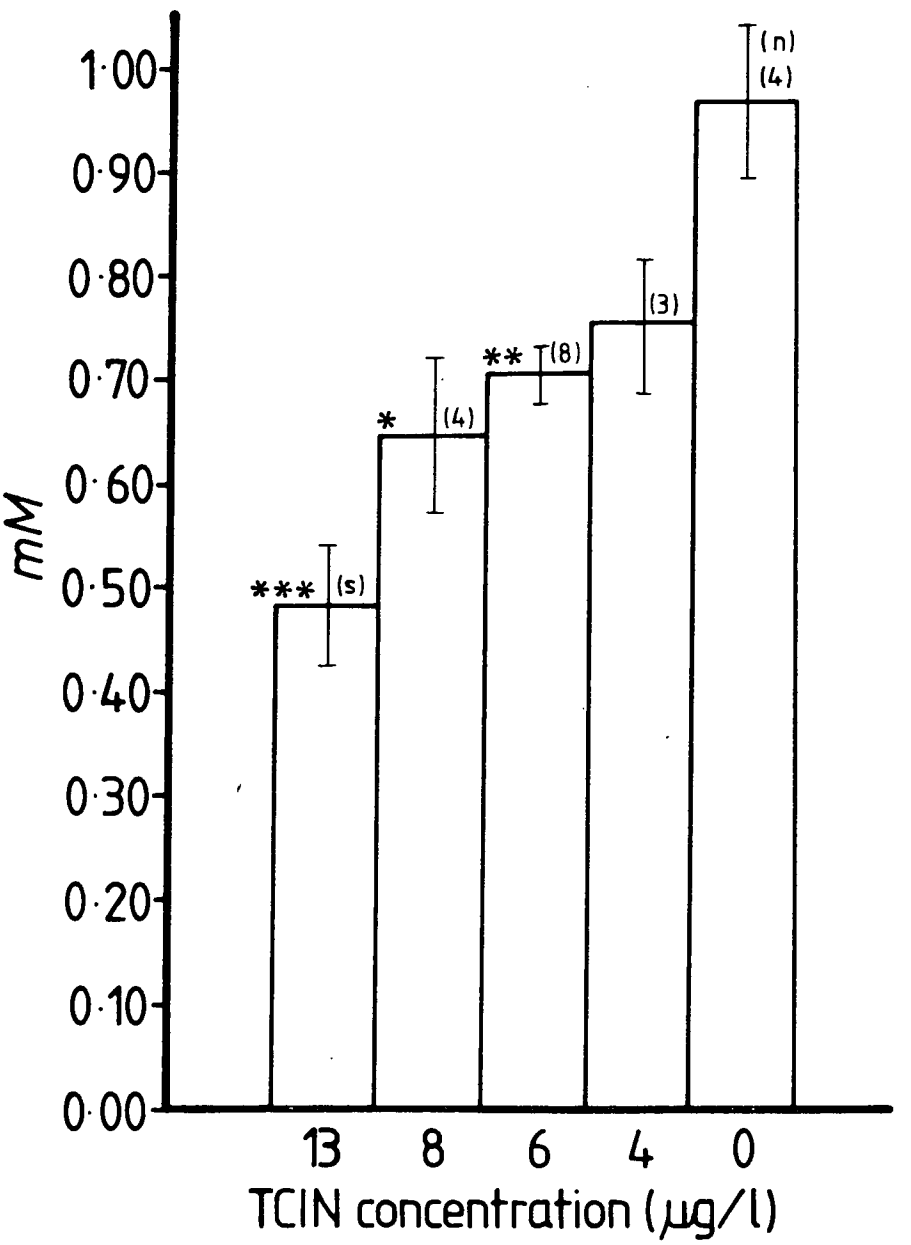


Fig. 9.1 Total soluble thiol levels of G. truttaceus liver after exposure for four days to varying levels of TCIN without feeding.

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

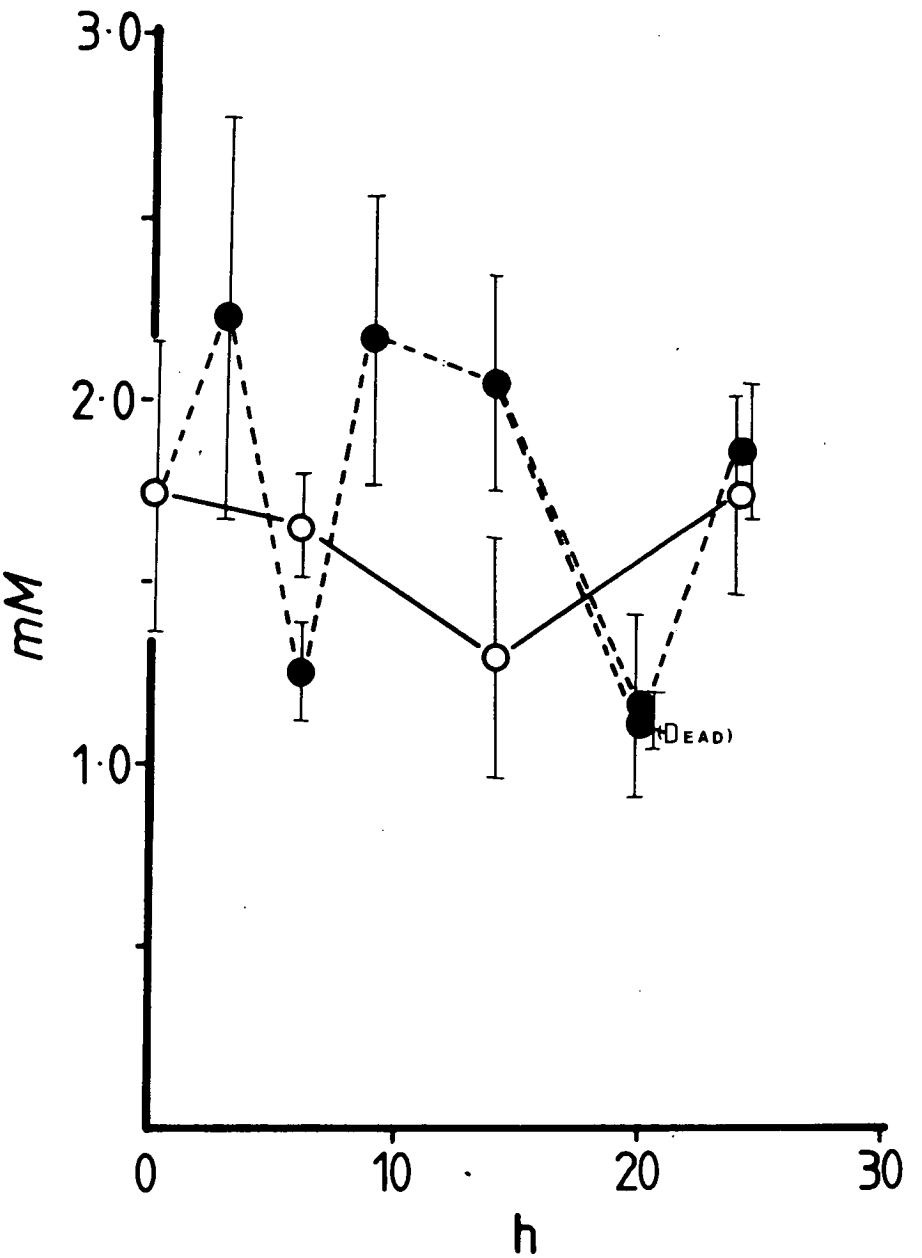


Fig. 9.2 Levels of GSH in livers of fed S. gairdneri over 30 h.

O = control fish.

● = fish exposed to 30 ug/l TCIN. (N = 4).

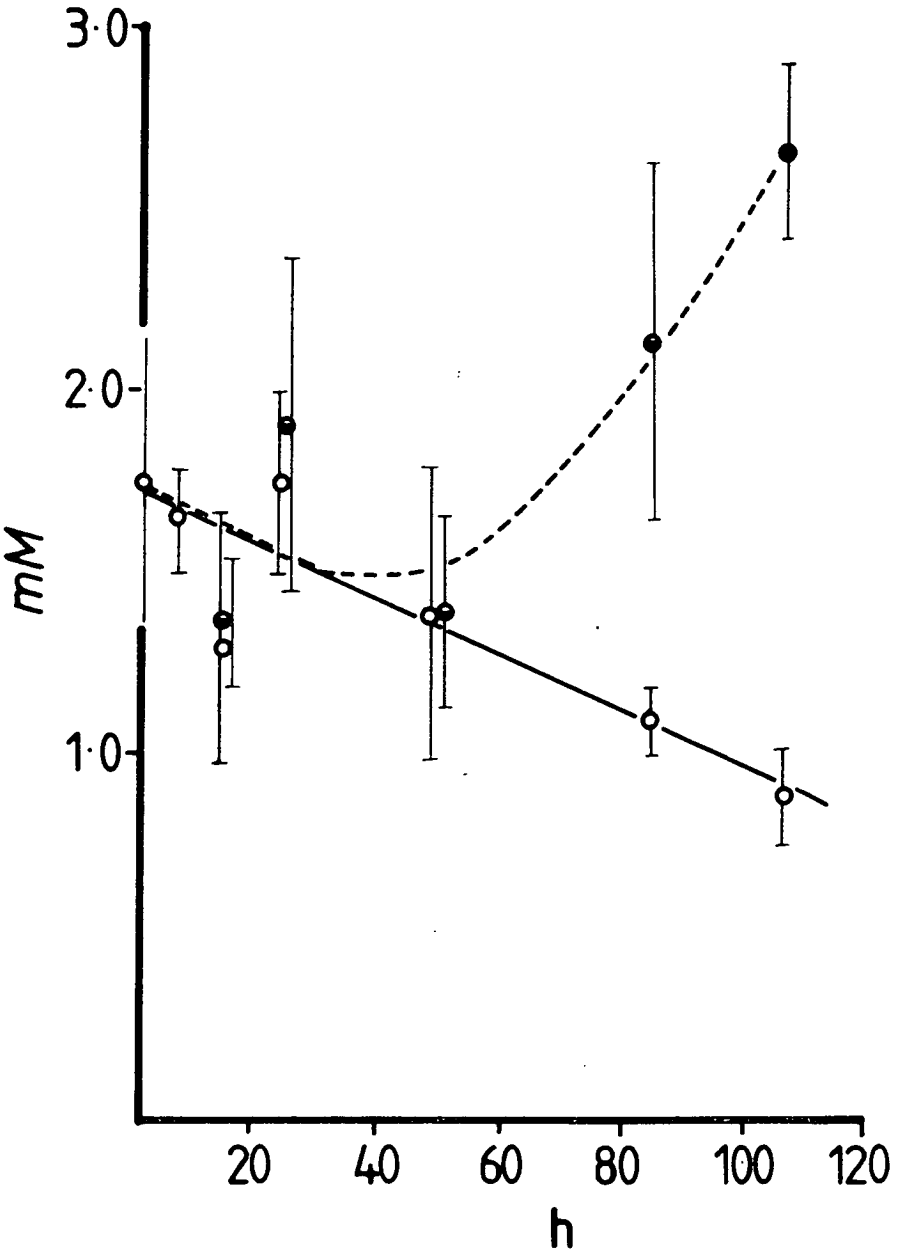


Fig. 9.3 Levels of GSH in livers of fed S. gairdneri over 120 h.

O = control fish.

⊖ = fish exposed to 10 ug/l TCIN. (N = 4).

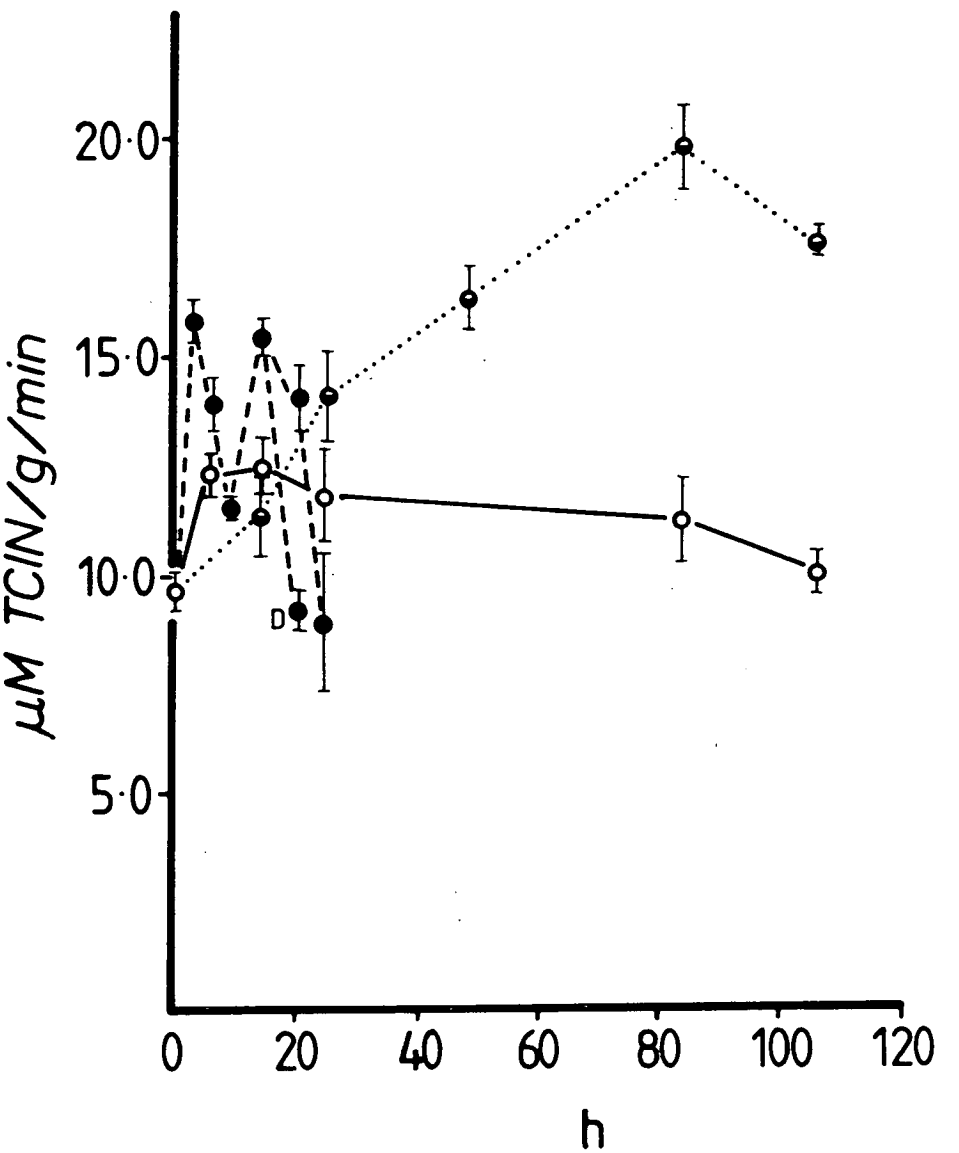


Fig. 9.4 S. gairderi hepatic GST activity toward CDNB over 120 h.

O = control fish.
● = fish exposed to 30 ug/l TCIN.
◒ = fish exposed to 10 ug/l TCIN. (N = 4).

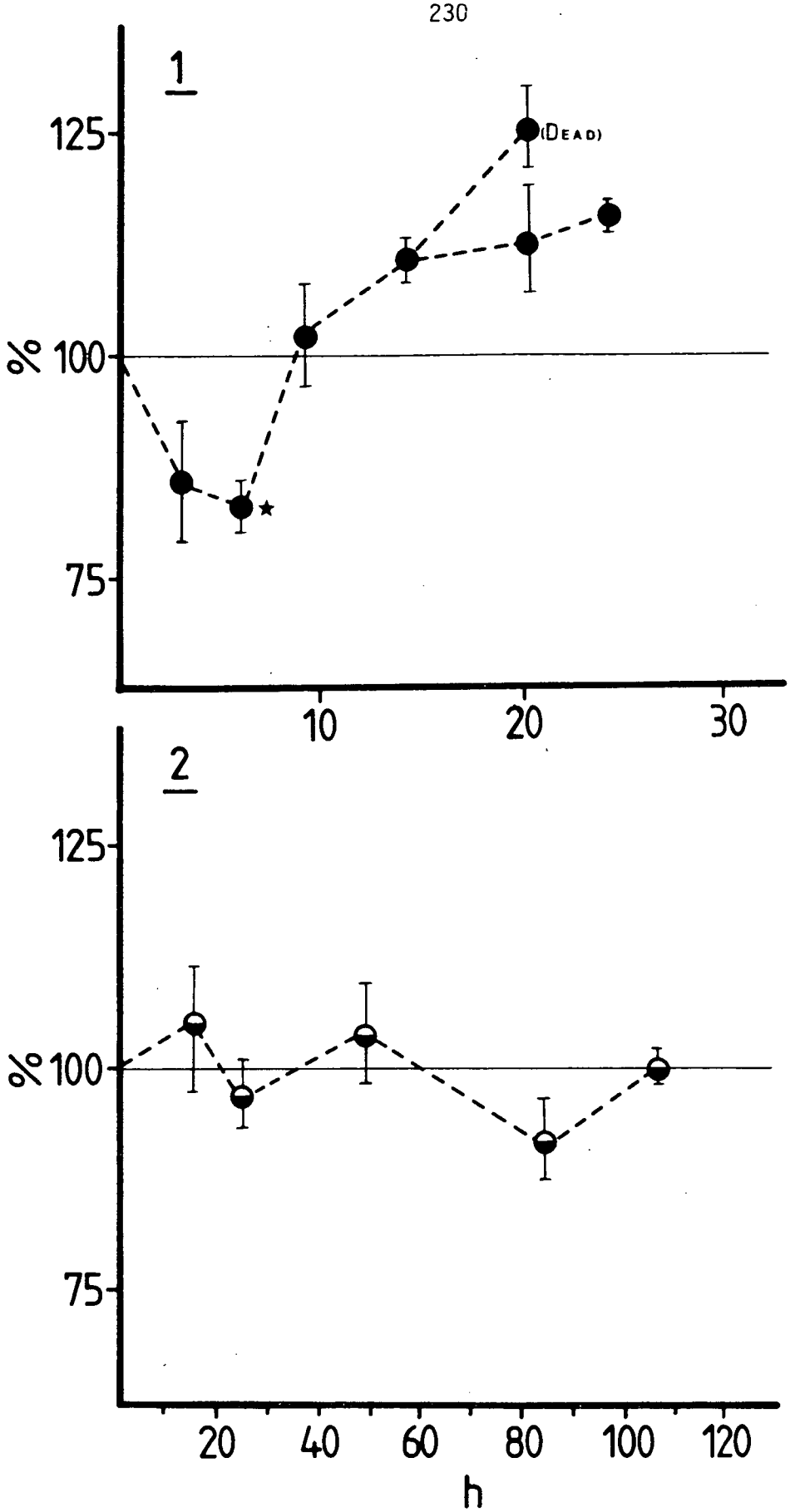


Fig. 9.5 GAPDH activity (% of controls) in livers of S. gairderi exposed to 30 (1) and 10 (2) ug/l TCIN. * = $p < 0.05$.

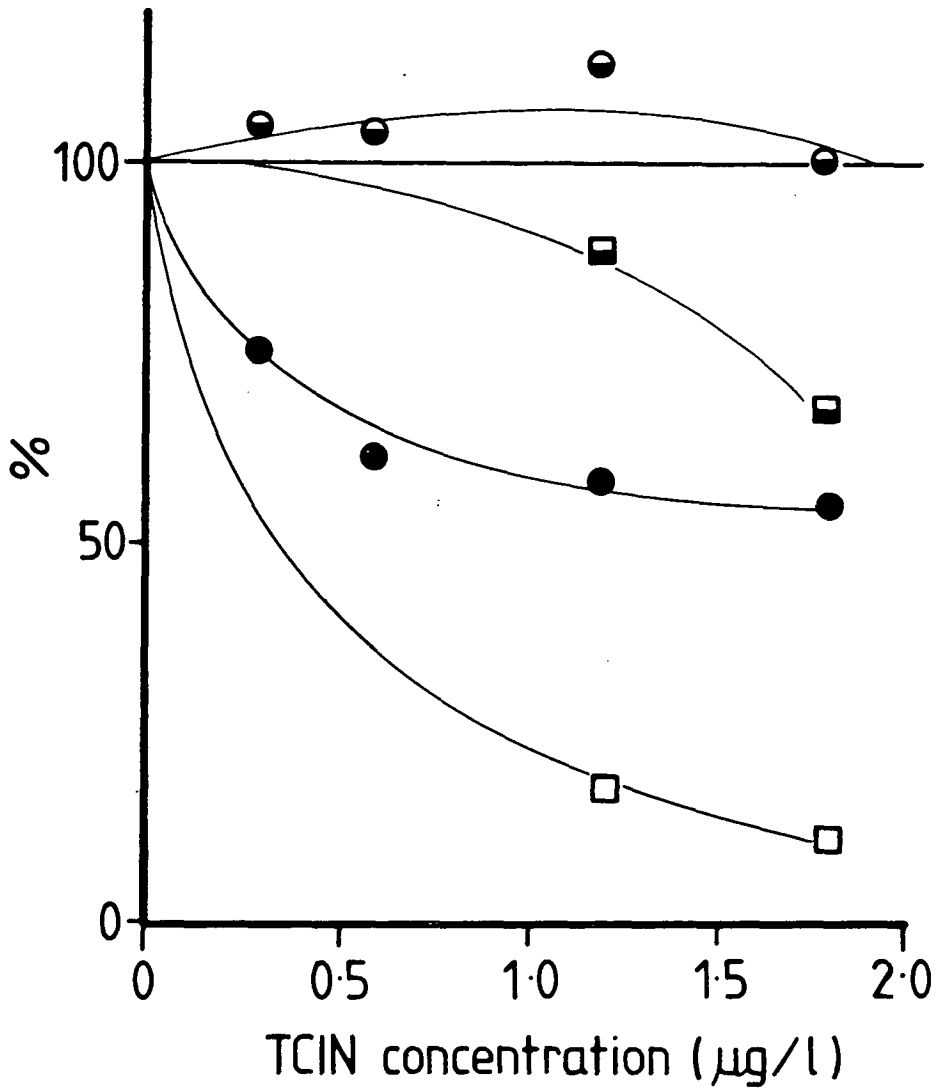


Fig. 9.6 GAPDH activity (% of control values) in in vitro assay preparations after exposure to varying concentrations of TCIN for 25h.

◒ = S. gairdneri hepatic cytosol at 0.516 nmole/ml GAPDH assay concentration.

● = S. gairdneri hepatic cytosol at 0.017 nmole/ml GAPDH assay concentration.

□ = Pure GAPDH at 0.017 nmole/ml assay concentration

◪ = Pure GAPDH at 0.017 nmole/ml assay concentration with 1 mM GSH. (N = 3).

exposure for 2.5 h agreed with the results of Long and Siegel (1975), where the concentration of GAPDH and TCIN were similar to those used here. They obtained 81% inhibition of GAPDH on 2.5 h exposure to 1.0 ug/l TCIN, which is comparable to the 82% inhibition found here (Fig. 9.6). On addition of 1 mM GSH, inhibition of GAPDH by TCIN was markedly decreased. An attempt to show the effect of GSH removal in cytosol preparations on TCIN inhibition of GAPDH, was unsuccessful due to the interference of the colour reaction of DTNB with GSH, in the spectrophotometric GAPDH assay.

9.1.4. DISCUSSION

The rapid conjugation of TCIN by GSH, catalyzed by GSH leads to a build up of metabolites in the bile of unfed fish (Chapter 6). There are two possible roles for GSH in the toxic action of TCIN in fish. Since GSH conjugation results in the formation of highly polar glutathione and mercapturate metabolites, regarded as less toxic and easily excretable, GSH may be acting in a protective detoxication role.

TCIN was observed to cause depletion of GSH reserves in cells of the fungus Saccharomyces pastorianus (Vincent and Sisler, 1968); this is considered to be the primary cause of fungitoxicity. If significant depletion of GSH occurred in TCIN exposed fish, then the GSH conjugation reaction may play a primary role in causing toxicosis.

The effect of TCIN exposure on hepatic GSH levels in fish studied in this work is related to feeding. Glutathione levels in mammals are also known to be related to feeding status (Arias et al., 1982). The decrease in hepatic total soluble thiol in unfed G. truttaceus indicated a dose-related response. It appears that TCIN conjugation causes a depletion in GSH levels which is not corrected by increased GSH production, probably due to lack of nutritional input.

By contrast Salmo gairdneri, fed during sublethal TCIN exposure, responded by increasing both GSH and GST levels. These responses would cause an increase in the GSH-TCIN conjugation rate both due to increased substrate concentration and catalytic activity. No such response occurs with fish exposed to a lethal concentration. Time for initiation of inductive processes may have been too short. Since GSH levels do not decrease at lethal exposure levels, it appears

that the GSH-TCIN reaction plays a protective detoxication role. The rate of conjugative detoxication at 30 ug/l TCIN is insufficient to prevent toxicosis.

Until recently, G S-transferase enzymes were regarded as not being inducible. However, Kaplowitz et al. (1975) showed that MFO inducing agents will also induce GST activity. TCDD has also been shown to induce GST (Baars et al., 1978). DDT pretreatment of rats induced GST activity to four different substrates, but induction only occurred toward one substrate in non-human primates (Down and Chasseaud, 1979), indicating species-species variability in the response. No work has been reported on the dynamics of GST activity in fish, with the exception of the report of an absence of effects on GST activity in sheepshead minnows by exposure to PCB when MFO activity was induced (James and Weatherby, 1981). The well-known ability of GST enzymes to bind, sometimes sacrificially, has been reported as a means to explain transient decreases in GST activity in rats on exposure to dibromoethane and CCl_4, which also caused GSH depletion (Botti et al., 1982). Decreases in GST activity, although observed in the exposures of S. gairdneri to TCIN at 30 ug/l, were not sufficient to support a hypothesis of failure of detoxication by loss of GST activity due to binding. Binding in vivo may have been inhibited by the high levels of GSH that were maintained, even during lethal exposure.

Induction of several drug-metabolizing enzymes in rats on exposure to m-dichlorobenzene was attributed to 3,5-dichlorophenyl-methylsulphone, a sulphur metabolite of the parent compound (Kimura et al., 1983). Such a sulphur metabolite, derived by secondary metabolism of glutathione and mercapturate conjugates, may well be responsible for the induction of GST activity on TCIN exposure. It should also be noted here that such secondary sulphur metabolites are known to be toxic and mutagenic in their own right (Koss et al., 1977; Reed and Beatty, 1980). This effect is almost certainly determined by TCIN flux through the liver.

The addition of GSH to the medium in which pure GAPDH was exposed to TCIN caused a marked decrease in inhibition (Fig. 9.6). This indicates that, without catalysis, GSH competes more effectively for available TCIN than does GAPDH. This may be related to both the pKa and steric environment of the thiol functions of GSH and the

GAPDH active site, although a concentration effect is active here. The liver concentrations of GSH and GAPDH measured in this work were 1 - 2 mM and 40 mM respectively. In cytosol exposed TCIN, the GAPDH activity was markedly higher than in the pure GAPDH preparations, when the GAPDH and TCIN concentrations were the same for both assays (Fig. 9.6). This is not unexpected given the efficiency with which cytosolic GST catalytically removes TCIN by GSH conjugation.

The GST mediated GSH conjugation reaction, therefore, plays a protective role by decreasing TCIN available for protein binding. The induction of GST and GSH levels on sublethal TCIN exposure may act to prevent protein-binding by TCIN from increasing with time, as observed in vivo (Chapter 6).

Hexachlorobenzene (HCB) has been shown to be metabolised to mercapturate and other sulphur metabolites, after glutathione conjugation, in mammals (Koss et al., 1977). HCB causes toxic porphyria, a condition involving disturbance of porphyrin metabolism, and concomitant haemolytic anaemia. TCIN causes haemolytic anaemia in exposed fish (Chapter 4). It is possible that secondary metabolism of glutathione conjugates of TCIN may produce a thiophenolic analogue of pentachlorothiophenol, a metabolite of HCB believed to lead to the condition of porphyria. This metabolite was not detected in fish. Hepatic and urinary levels of porphyrins were not analysed, but the lack of secondary porphyria symptoms characteristic of the condition (Granick and Urata, 1963): red fluorescence of gallbladder, hind-gut and liver under ultraviolet light, and release of red porphyrins in the urine, indicates that toxic porphyria is not a condition elicited by TCIN exposure.

TCIN conjugates up to three molecules of GSH (Vincent and Sisler, 1968). It is difficult to assess the relative roles of the fish biliary mono- and di-conjugates in the toxic action of TCIN since some degree of hepatic recycling is inevitable (Arias et al., 1982). The electrophilic nature of TCIN would be reduced by GSH conjugation, but it is not possible to discount secondary or tertiary conjugation by protein thiol groups. The pharmacokinetic behaviour of TCIN is complicated by this ability to multiply bind thiol residues, as well as by the induction of hepatic GST and GSH levels.

CHAPTER 10

COMPARATIVE ASPECTS OF DETOXICATION

10.1 COMPARATIVE ASPECTS OF DETOXICATION

10.1.1 INTRODUCTION

Despite intensive research effort into mechanisms of xenobiotic detoxication, primarily in mammals (Jakoby et al., 1982), and efforts to correlate the degree of toxicological responses with the chemical structure or physical properties of toxins (Sabljic, 1982; Veith et al., 1983; Gottschall et al., 1983), little effort has been made to relate the degree of toxicological responses with the efficiency of detoxication within the organism. The investigation of direct relationships between the toxicity of toxins and the detoxication is complicated, for a number of reasons (Fig. 10.1).

There are two types of detoxication enzyme systems; these are detailed in Table 10.1. Many compounds, especially aryl and unsaturated alkyl hydrocarbons are metabolized by way of phase I enzymes. The products of phase I metabolism are not always "detoxified" compared to the parent compound. Indeed, the epoxide products of the cytochrome P450 monooxygenase system are very reactive and many are widely regarded as primary carcinogens due to their ability to react with macromolecules including DNA (Ahokas et al., 1979). This has been shown to occur in Salmo gairdneri after administration with aflatoxins (Stott and Sinnhuber, 1979). Phase I metabolites, unless reacted with other cellular constituents, are often subjected to secondary metabolism mediated by the Phase II enzyme system, and usually consisting of conjugation of an electrophilic, phenolic or other polar site with a polar residue.

This second phase is often regarded as genuine detoxication since most conjugated metabolites are less toxic and are readily excreted, as they are generally more polar than the parent compound. Even here, however, secondary complications may arise. Conjugate products of the phase II system may give rise to tertiary products of

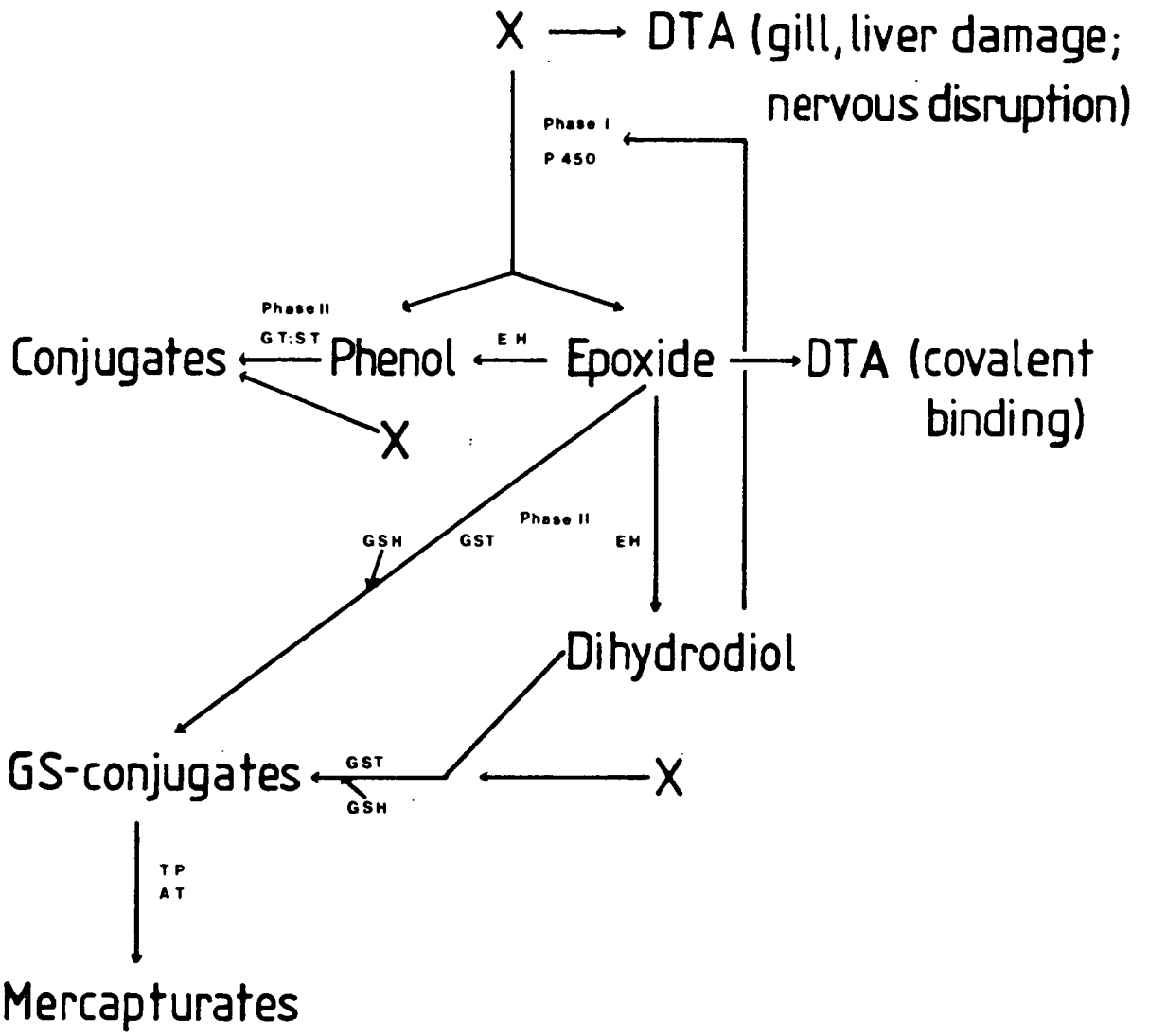


Fig. 10.1 General scheme for metabolism and toxic action of organic xenobiotics, X. DTA = direct toxic action. P450 = cytochrome P450 system: GT = glucotransferase. ST = sulphotransferase. GST = glutathione S-transferase. GSH = glutathione. TP = transpeptidase. AT = acetyl transferase.

Table 10.1 Principal detoxication enzyme systems

Enzyme System	Phase	Pollutant Type	Metabolic Process
Cytochrome P450 (Benzopyrene hydroxylase)	I	Aryl & alkyl hydrocarbons	Oxidation, epoxidation, dealkylation
Epoxide hydrolase	I	As above	Hydration of epoxides from 1
Glucuronosyl transferase	II	Phenolics, ROH, RCOOH	Glucuronide conjugation
Glutathione S-transferase	II	Aryl halogen, epoxides	Glutathione conjugation
N-acetyl transferase	II	Amines, amino acids	Acetylation
Sulfotransferase	II	As for 3	Sulphate conjugation

high toxicity. This is often a function of the excretory route which proceeds via the bile. Intestinal microflora can metabolize mercaptan conjugates to thiophenolic coumpounds which, on resorption into the body, may produce secondary toxic effects (Koss et al., 1977). Also, since phase II detoxication involves a secondary substrate, significant depletion of that substrate may result in toxic effects. This is generally considered to be a significant factor only with glutathione, and has been discussed elsewhere in this thesis with reference to TCIN (Chapter 9). A general scheme proposed for metabolism of organic xenobiotics is shown in Fig. 10.1, with possible toxic repercussions being noted.

There are a number of classes of xenobiotics in which the dynamics of detoxication are relatively simple, and where metabolically induced secondary toxicological effects are few or non-existent. These are toxins where metabolism is primarily dependent on the phase II system, and includes phenols, some organophosphates, and readily conjugatable electrophiles. Such compounds could be used to test hypotheses of detoxication mechanisms by correlations of toxicological data and enzyme activities.

TCIN is such a compound. It was decided to see if a correlation existed between the toxicological data for TCIN, described in Chapter 3, and levels of GSH and GST activity in Salmo gairdneri, G. maculatus and G. auratus, obtained under standard conditions.

10.1.2 MATERIALS AND METHODS

Fish collection and acclimation

Salmo gairdneri were provided by Sevrup Fisheries Pty. Ltd., Bridport, Tasmania. Galaxias maculatus and G. auratus were collected by electrofishing in previously described locations (3.1.2). Eight indivuals of each species were selected to fall within the same weight range, around 8.0 - 9.0 g.

All fish were held in flow-through aquaria at 16° C, as previously described (3.1.2) for 21 days. They were fed daily to satiation on chopped chicken liver. At the end of the holding period all of the fish were killed (tricaine methanosulfonate), and their livers excised and weighed.

Analysis of hepatic cytosol

Livers were homogenized in 8 vol. 0.1 M Tris-HCl (pH 8.3) buffer and centrifuged at 30,000 g for 1 h. The homogenates were snap-frozen and stored in liquid nitrogen until assayed.

GSH was assayed by the method of Ellmann (1956), with $HClO_4$ deproteination. GST activity was assayed using CDNB substrate by the method of Habig et al., (1974), and C^{14}-TCIN by the method described in 8.1.2 at concentrations of 150 and 1 uM CDNB&C^{14}-TCIN respectively.

Liver-body weight regressions

Livers (N = 25-50) of fish of each species were weighed and plotted against body weight.

10.1.3 RESULTS

Levels of GSH and GST activity for the two substrates are shown in Table 10.2 for the three species, along with hepatic content values corrected using the liver-body weight regressions for 9.0 g fish.

The orders of the different parameters are shown in Table 10.3 along with predicted orders of toxic response, when high levels of GST activity are considered to promote detoxication. Experimentally determined orders of toxic response to these species are also shown in Table 10.3, from Chapter 3 for 96 h LC50 values of TCIN. The species orders of total GST activity toward C^{14}-TCIN and of asymptotic LC50 values were in agreement.

10.1.4 DISCUSSION

Tanaka et al., (1981) correlated inherited differences in resistance to lindane and hexadeuterated lindane in strains of housefly with the ability to detoxify these compounds, measured as GST and oxygenase activities. This study shows that measures of detoxication rates in species of fish could be correlated with lethal responses of the species of toxins.

It is known that the GST-GSH conjugation of TCIN in fish constitutes a detoxication reaction (Chapter 9), whereas in fungi it is considered to be the primary cause of toxicity through cellular GSH

Table 10.2 Species levels of hepatic GST and GSH
A = hepatic tissue concentration
B = liver wt. corrected total values (9 g fish)

Species	Wt (SD) g	N	GST A: umol/min/g B: umol/min x 10^{-3} CDNB	GST C^{14}-TCIN	GSH A: umol/g B: umol
S. gairdneri	9.45 (1.13)	8	A: 0.1140 B: 10.740	0.2380 22.422	3.797 0.358
G. maculatus	7.84 (0.86)	8	A: 0.1813 B: 25.506	0.2782 39.142	2.694 0.379
G. auratus	9.83 (0.71)	8	A: 0.0585 B: 14.329	0.2448 60.000	1.568 0.384

Table 10.3 Species orders of hepatic GST activity and LC50 responses to TCIN

	Substrate	Species order
GST activity content	C^{14}-TCIN	G. auratus > G. maculatus > S. gairdneri
	CDNB	G. maculatus > G. auratus > S. gairdneri
GST activity per g liver	C^{14}-TCIN	G. maculatus > G. auratus > S. gairdneri
	CDNB	G. maculatus > S. gairdneri > G. auratus
96 h LC50	TCIN	G. auratus > S. gairdneri ≥ G. maculatus
Asymptotic LC50	TCIN	G. auratus > G. maculatus > S. gairdneri

depletion (Vincent and Sisler, 1968). The results given here show that the available hepatic free GSH reserves in the three species studied do not differ significantly. It should be noted that these results depend on a comparison of animals maintained under the same conditions, and at optimal nutritional condition. Very few studies of GSH levels in fish have been made. Huillet et al., (1970) found that tissue GSH levels in the scorpion fish, Scorpaena porcus, decreased under conditions of stress, such as increases in water potassium concentration, low oxygen levels and decreased osmotic pressure. This decrease was believed to be related to reductions in the rate of glucose metabolism under stress. Glutathione levels are also known to be reduced under starvation (Meister, 1982). Consequently, any comparison of species of GSH levels should be made under standard conditions with optimal feeding.

The total hepatic GST activity differed markedly from species to species, and depended markedly on substrate type. The activity of GST toward C^{14}-TCIN gave the best correlation with the order of asymptotic LC50 values for TCIN in the species studied. Since liver GSH contents did not differ, they had no influence on the correlation of detoxication efficiency with LC50 values. The work in Chapter 9 demonstrated that low level exposure to TCIN caused marked induction in hepatic GSH and GST levels. Consequently species differences in the extent of chronic effects of TCIN may well be influenced by the relative ability of species to induce GST and GSH. No effects on hepatic GST or GSH were found on lethal exposure to TCIN, however.

10.2 PROPOSAL FOR A DETOXICATION SCREENING PROCEDURE

As indicated in Chapter 1, very little single species or systems aquatic toxicological research has been undertaken with organic xenobiotics in Australia (AWRC, 1982), despite intensive research efforts elsewhere. The native fish fauna, of which some 90 - 100 species occur in areas likely to be exposed to agricultural and industrial pollutants, is, therefore, dependent on standards set for those species researched overseas.

If detoxication enzymes systems are the principal means of disposal of organic xenobiotics, then relative levels of these systems

and their secondary substrates in fish species should determine the relative susceptibility of species to such toxins. The results of the previous section support this hypothesis. It would be useful, therefore, if the native Australian fish fauna were screened for species susceptibility to classes of toxins by examining the activity of detoxication enxymes toward those toxins, as a preliminary step in toxicity testing studies.

There are several advantages in such a screening procedure. Species most susceptible to broad types of organic pollutants can be selected for detailed toxicity testing in order to set water quality standards relevant to the most susceptible fish species. The preliminary selection process avoids the use of large numbers of fish, often greater than 100, in expensive, single compound toxicity tests which are of little general utility. The screening procedure can involve small fish samples (10-20), and detoxication enzyme assays can be performed on the same samples for a wide range of pollutant classes at a variety of temperatures. Screening of samples of different sexes, life cyle stages and populations would be simple and rapid. The assay techniques for all the major detoxication enzyme systems are well established, although care must be taken to use appropriate substrates since the enzymes, though broad in specificity show marked variation in activity dependent on substrate type (Gregus et al., 1983).

There are, however, several cautionary notes. As discussed in some detail in the introduction to the previous section (10.1.1), the relationship between xenobiotic enzyme detoxication activities and lethal or chronic responses is complex, and has not been researched to any great extent in fish. More pilot studies of the type performed in section 10.1 are needed in order to give a firm foundation to the knowledge of the relationships in other classes of pollutants, especially phase I metabolizable compounds (polyaromatic hydrocarbons, PCB's etc.).

This study introduces the suggestion that the native Australian fish fauna could be screened for species susceptibility to classes of toxins by examining the activity of detoxication enzymes, as a preliminary step in toxicity testing studies. Great care must be taken in examining such a possibility for several reasons. Firstly, despite there existing a wealth of information on assay and preparative

techniques for studying these enzyme systems (Viz. Gregus et al., 1983), little concrete information exists on the relationships between the action and activity of detoxication enzymes and the toxicology of the substrate toxins, especially in fish species. Secondarily, more information is required on the relationships between sex, body size, lifecycle stage, nutritional state and effects of environmental variables on detoxication enzyme activities. Koivisaari et al. (1981) demonstrated seasonal and sexual variations in the cytochrome P450 content and monoxygenase activities (Phase I), and in glucuronidation activity (Phase II) in Salmo gairdneri. Such information could be incorporated into studies in order to select species at risk to classes of pollutants by examining organ levels of detoxication enzymes, extending the utility of species studies like that of Lindström-Seppä et al. (1981 a) on the vendace, Coregonus albula. Choise of substrate in enzyme assays appears to be important and should preferably represent the class of toxicant to be assessed as closely as possible (Gregus et al., 1983). Also, although it appears that hepatic levels of phase I and phase II detoxication enzymes are generally highest, and could possibly be used as relative indicators of species detoxication ability, it should be noted that extrahepatic metabolism is also significant (Lindström-Seppä et al., 1981 b).

If such a screening procedure were implemented, it would, along with considerations of economic importance, distribution, population stability and status on the endangered species list (Fish Biol. Soc. of Australia), allow selection of particular species for intensive toxicological study and from which water quality standards coud be proposed.

CHAPTER 11

GENERAL CONCLUSIONS

This study has attempted to:

a) Assess the risk of environmental exposure to TCIN for freshwater aquatic organisms. This has been done in 3 ways:

   i) Examining environmental levels after aerial spraying.

   ii) Examining the biodegradation of TCIN.

   iii) Examining the stream dynamics of TCIN.

b) Assess the toxicity of TCIN to a commercially important fish species Salmo gairdneri, and three galaxid species: two, Galaxias maculatus and G. truttaceus which occur in environments likely to be contaminated in Australia, and one Galaxias auratus, a species on the endangered species list of the Australian Society for Fish Biology, which occurs in an unpolluted environment and was studied for comparative toxicological and biochemical purposes.

c) Examine the chronic effects of TCIN in Salmo gairdneri.

d) Examine the pharmacokinetics of TCIN in organs of Salmo gairdneri using C^{14}-TCIN, synthesized during the course of this study.

e) Examine the route of primary metabolism of C^{14}-TCIN.

f) Study the enzymatics of TCIN detoxication in all the species above, and including Salmo trutta.

g) Attempt to uncover the means of toxic action of TCIN and the role of the GSH - GST conjugation reaction in that action.

h) Test the hypothesis that the activities of detoxication enzymes can determine the level of a species' toxic responses to a toxicant.

TCIN was found in streams after aerial spraying at concentrations between 0 and 5 ug/l. On the basis of subsequent experiments, a relationship between TCIN toxicity and oxygen levels was found. Low summer oxygen concentration in 1 - 2 year ponds of the Bridport and Muddy Creek trout farm could, therefore, enhance the response to low TCIN levels, and may lead to the observed stress behaviour. TCIN levels observed in the streams would not cause a respiratory response, based on experiments with a 2 h response time, although low oxygen levels could lower the concentration at which such a response is elicited.

TCIN disappears relatively rapidly in stream water by a combination of adsorption and conversion to polar forms, probably DAC3701. The toxicity of the latter compound to fish is much lower than TCIN, and it is unlikely to occur at levels likely to instigate toxic responses in fish. TCIN has a high affinity for suspended organic material and sediment-adsorbed TCIN comprises a high percentage of stream contamination. TCIN is rapidly stripped from stream water, probably by sediment-association and surface adsorption. This behaviour is consistent with predictions from correlations with the octanol-water partition coefficient of TCIN.

TCIN is highly toxic to fish. It is more toxic than closely related compounds such as dichlobenil, pentachlorophenol (PCP) and lindane. Salmo gairdneri, Galaxias maculatus, G. truttaceus and G. auratus all have 96 h LC_{50} values around 15 - 20 ug/l for TCIN, and no large differences occurred in lethal response levels between Salmo and galaxiid fish. Salmo gairdneri showed significant changes in ventilation frequency above 20 ug/l over a 2 h response period. The magnitude of these changes was dose-dependent, the threshold of response being approximately 50 ug/l over a 30 min response period.

TCIN caused chronic changes in gill morphology and bile pigmentation and led to a dose-dependent haemolytic anaemia. It was bioconcentrated with a whole body 24 day BCF value of 13.3 times, inconsistent with the values expected from the octanol-water partition coefficient. This result and the observation of the lack of detectable parent residues in fish exposed to lethal levels of TCIN indicated that the compound was rapidly metabolized or excreted.

Metabolism studies in Salmo gairdneri using C^{14}-TCIN showed that TCIN is readily metabolised to polar forms and excreted by fish. The principal site of TCIN metabolite build-up is the bile, in which some 65 - 70% of biliary residues consisted of TCIN-glutathione conjugates. These conjugates were readily degraded by biliary enzymes, probably by way of the cysteine derivatives. Approximately 50% of TCIN residues in the liver were protein bound. Less than 1% was protein bound in the bile. Binding of TCIN to metallothionein was not a significant protein-binding detoxication route, a result consistent with similar mammalian studies, even after zinc exposure had induced metallothionein.

The conjugation of TCIN to glutathione (GSH) was catalyzed by enzymes occuring in the cytosol of a number of organs, especially the liver. The bile did not show this catalytic ability. The properties of these enzymes were consistent with those of the glutathione S-transferase (GST) group. The existence of GST was confirmed in Salmo gairdneri, S. trutta, and the galaxiid species studied above. The existence of at least two GST enzymes was confirmed in all species. The molecular weight of the GST with peak activity toward TCIN was consistently higher (5, - 10,000) than that with peak activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in all species. The GST enzymes of peak activity toward both substrates were consistently lower by 10, - 20,000 in the galaxiid species than in the two salmonids, indicating a major phylogenetic difference in amino acid composition of the GST enzymes. pH profiles showed peak GST activity at pH 8.

G. maculatus GST was studied in some detail. TCIN was found to bind covalently to and inhibit GST when GSH was absent. The rate of conversion of TCIN to polar metabolites by liver cytosol was dependent on the presence of free GSH. The GST catalyzed GSH - TCIN conjugation reaction was found to be first order in TCIN and its conjugate

product. Although assay reaction rates for CDNB and TCIN with GSH were similar, TCIN reaction rates were limited by its low water solubility. When compared independent of concentration, the GST catalyzed TCIN - GSH reaction was 6 x 10^3 times faster than that of CDNB. Since the uncatalyzed reaction of TCIN was only 20 times faster than that of CDNB, some preferential "activation" of TCIN must occur in the binding site of GST. Acephate had no effect on GST activity toward TCIN.

TCIN decreased hepatic soluble thiol levels in unfed G. truttaceus. GSH and GST levels increased with time of exposure to 10 ug/l TCIN in fed S. gairdneri, but were unaffected by 30 ug/l exposure. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), although inhibited by TCIN at low pure enzyme levels in vitro, showed much lower inhibition in vitro with hepatic cytosol preparations and in the presence of glutathione. S. gairdneri GAPDH was not affected by 10 ug/l TCIN exposure in vivo, and showed only a transient decrease with exposure to 30 ug/l.

It, therefore, appears that the model shown in Fig. 11.1 can be suggested for the toxic action and detoxication of TCIN in fish, where GST mediated GSH - TCIN conjugation is established as playing the major role in detoxication.

Asymptotic LC50 values for TCIN could be best correlated with total hepatic GST activity toward TCIN in S. gairdneri, G. maculatus and G. auratus when compared under standard conditions. This led to the proposal of a screening procedure for Australian native freshwater fish in toxicological studies, using the activities of detoxication enzymes.

It appears that TCIN is metabolized by the glutathione-mercapturate pathway in both Salmo and Galaxias species. This pathway appears to act as a genuine route of detoxication, protecting proteins from further binding by TCIN. This is in contrast to the glutathione conjugation of TCIN in fungi, where that reaction acts as a major cause of toxication by depletion of GSH levels (Vincent and Sisler, 1968). This difference in role of the same reaction in different organisms is probably related to the large available thiol pool in fish with relatively low influx of TCIN per unit weight limited by input only at the gills. However, the low LC50 levels indicate that the GSH detoxication reaction is not highly effective in its ability to detoxify

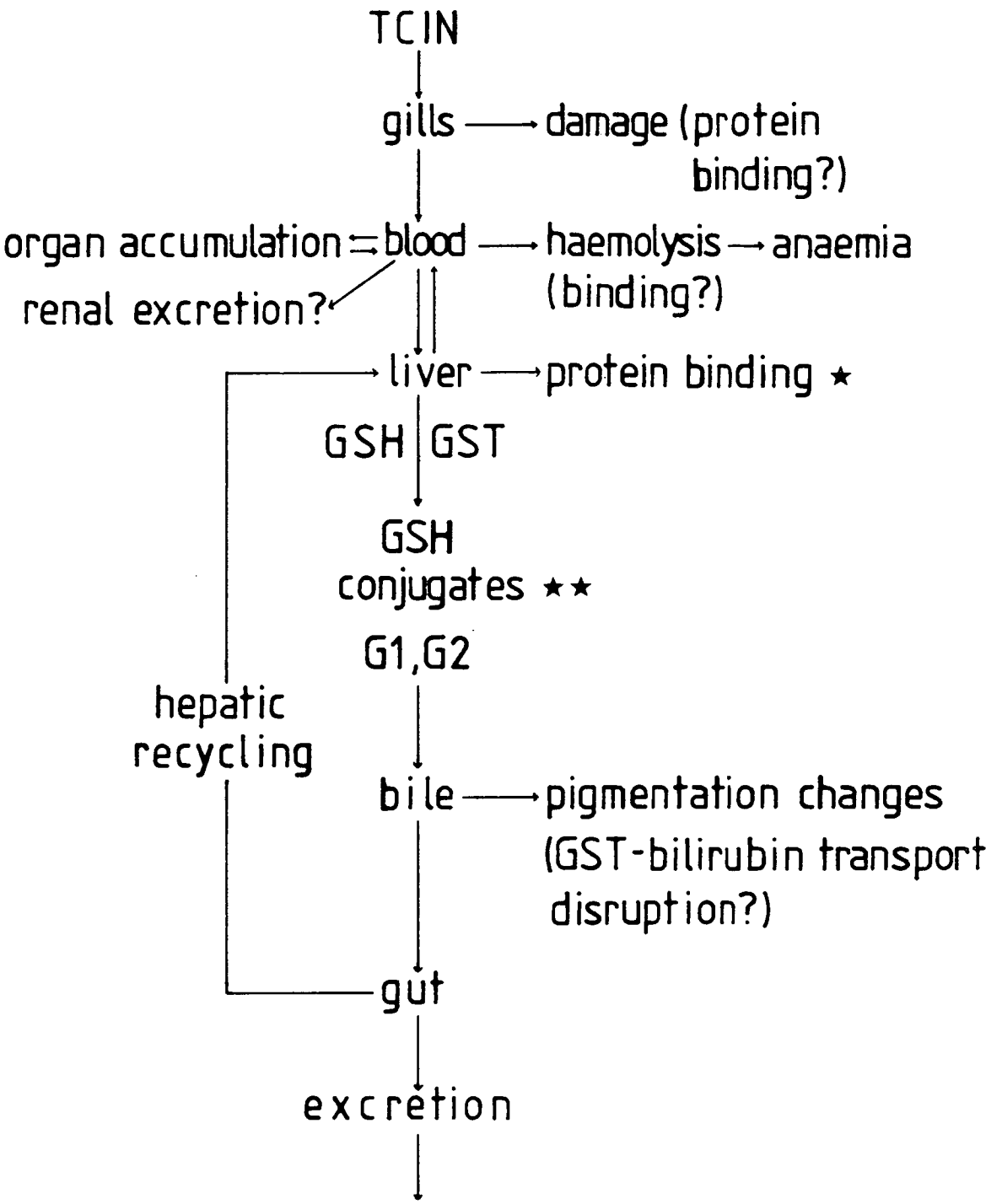


Fig. 11.1 Scheme of TCIN metabolism in Salmo gairdneri.

* = main toxic action.

** = main detoxication route.

the highly reactive electrophilic and multiply conjugating compound TCIN.

Despite relatively large differences in hepatic GST activity between Salmo gairdneri and Galaxias maculatus, differences in the LC50 values for TCIN were small. This indicates that secondary effects may have a significant bearing on the toxic action of TCIN even at varying levels of GST activity in the liver. It may be that hepatic GST activity does not completely account for the detoxication "status" of the organism, especially as significant GST activity was observed in other organs. Bauermeister et al. (1983) noted GST activity in the gills of S. gairdneri. Some primary conjugation may, therefore, occur at the gills. This was not investigated in this study. Blood plasma levels of TCIN residues were well above water solubility of TCIN. This may be due to conjugates formed by the action of gill GST as well as some protein binding. The appearance of di-conjugates in the bile may be due to primary conjugation at the gills followed by secondary conjugation at the liver. However, a secondary conjugation could also occur in the liver, and may be enhanced by hepatic recycling. The histological effects at the gills may be due to chronic protein binding by TCIN. It is unlikely that sustained GSH depletion is the cause since animals were exposed with feeding, and increases in hepatic GSH production observed at low level exposure would flow on into the blood pool and, consequently, the gills.

Changes in biliary pigmentation caused by TCIN exposure were not a result of toxic porphyria. They may have been caused by two mechanisms. Haemolytic anaemia caused by TCIN, may lead to enhanced excretion of haem breakdown products into the bile. G S-transferases are known to perform a function in hepatic transfer of bilirubin. Competition for GST binding sites has been demonstrated between bilirubin and GST conjugation substrates in vitro (Kamisake et al., 1975), and in vivo in mammals, especially as GSH conjugates (Barnhart and Combes, 1976). If it is occurring in fish, it may be that TCIN competes for binding sites in GST either during the course of the conjugation reaction, or by covalent binding, and upsets the transport of bile pigments, leading to changes in the make-up of bile.

In conclusion, this study provides interesting information on comparative pharmacological and toxicological processes, which occur in the salmonids and galaxiids. TCIN is an unusual compound due to its high electrophilicity and its ability to multiply conjugate cellular thiol residues, and its apparent immunity to phase I processes. This study indicates the importance of studying environmental toxicology in the context of low levels actually occurring in the field. Environmental contamination by organic xenobiotic chemicals does occur widely in Australia (Connell, 1974), and the native fish fauna and associated biota are widely subject to varying degrees of aquatic contamination. Although this study shows a similarity in response to one widely used agricultural chemical between the galaxiids and the ubiquitous test species Salmo gairdneri, this is by no means necessarily the case for the Australian fish fauna as a whole. Consequently, a greater research effort is needed in order to establish safety margins relevant to native aquatic fauna, in order to adequately protect the freshwater environment and to generate a greater awareness of aquatic chemical pollution and its effects on the biota in Australia.

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APPENDIX 1

PESTICIDES USED IN THE BRIDPORT - SCOTTSDALE AREA

CROP	TYPE	TRADE NAME	CHEMICAL
Potatoes	Pesticides	* Bravo	Chlorothalonil
		* Mancozeb	mancozeb
		* Orthene	acephate
		Thiodan	endosulphan
		Birlane	chlorfenvinphos
	Herbicides	Paraquat	gramoxone
		Diquat	reglone
Pastures	Pesticides		fenitrothion
		Birlane	chlorfenvinphos
		Septine 80	carbaryl
		* Lorsban	chlorpyrifos
		Metasystox	metasystox
	Herbicides	Estone 80	2-4-D Ester
		Methoxone	2-4-D Amine
		Butoxone	2-4-5- T
		Dicamba	
		MCPA	MCPA
Poppies	Herbicides	Barban	
		Paraquat	gramoxone
		Diquat	reglone
		Ramrod	propachlor
		Dachtal	chlorthal
Onions	Herbicides	Probe	methazole
		Linuron	linuron 50 or afalon
		Paraquat	gramoxone
		Diquat	reglone
Carrots	Herbicides	Gesamil	propazine
Parsnips		Linuron	linuron 50 or afalon
			prometryne
Peas	Herbicides	Sencor	metribuzin
		D.N.B.P.	dinoseb

Those marked * were found at the Bridport airstrip and aerially sprayed.

APPENDIX 2

PRINCIPLES OF OPERATION OF FLOW-THROUGH SERIAL DILUTOR TOXICANT DOSING SYSTEM (FIG. 3.1)

The system provides six 40 l flow-through screened aquaria for holding fish during acclimation periods and for exposing fish to five different concentrations of a toxicant and a control water supply. Tank flow-rates are continuously variable and dependent on the output of tubes used in the header-dilutor assembly, which may be adjusted with taps or clamps. Typical flow rates of 150 ml/min were used during experiments with up to 20 4-15 g fish per tank in this work, and 1-2 l/min during holding periods when up to 60 fish were held in a tank. Supplementary aeration may be provided if necessary. It was not used in TCIN exposure experiments due to the volatility of TCIN.

Toxicant stock solutions, stored in a drum (I), were pumped by peristaltic pump into the first chamber of the dilutor baffle tank (F), see inset M, Fig 3.1. The concentration of the first chamber, and consequently the first tank, is determined by the concentration of toxicant in the stock solution, the rate of the peristaltic pump, and the inflow rate of the dilutor's water from the large bore tube (E) descending from the dilutor header tank (D; inset 2x). Once the first tank concentration is set by the three variables above, the subsequent tank concentrations are determined by dilution ratios derived from the ratio of flow rates into and out of each baffle tank chanber.

As indicated in the inset M of Fig. 3.1, the inflow of water from the first dilution tube is 2 x , twice the flow of all other tubes. Consequently, after a flow of x has left the first chamber, to supply tank 1, there is a net flow x across the slot in the baffle into the next chamber, aided by the slant in the tank. This overflow is immediately mixed with an inflow x of clean water, and the toxicant is thus diluted to approximately 50%. An outflow x supplies tank 2 with this 50% diluted toxicant, and x flows across the next slot to chamber 3. This gets diluted to 50% toxicant concentration by the addition of inflow x of clean water from the header tank. Thus, tank

3 receives water with a concentration of toxicant 25% of that in tank 1.

This process continues across the baffle tank, causing the five exposure tanks to have the following relative concentrations in descending order: 100%, 50%, 25%, 13%, 6%. The sixth tank receives water from a feeder tube direct from the header tank, and hence serves as a control. The above dilution ratios are not the only available. The ratios may be changed by changing the relative inflows and outflows from the baffle chambers either using taps or tubes of different bore sizes. The expected concentrations can be calculated using the ratios of flow rates, noting that the inflow rate toxicant stock may also influence the dilution values. A series of calibration curves may be set up by using fluorescein and measuring the dilution ratios by absorbance measurements at 280 nm. It should be noted that these cannot be used if a toxicant is likely to be adsorbed from solution by the tank or tubing surfaces. In such cases, as with TCIN, there is no substitute for taking samples from the exposure tanks and analysing them for toxicant concentrations.

Once set up, the system operates with little maintenance - the only "variable" being the consistency of flow-rate from the peristaltic pump. The addition of the primary header tank (C) is essential in order to avoid the effects of drops in water supply flow rates which can lead to emptying of the secondary header tank (D) and subsequent disruption of the experiment.

During the course of this work, galaxiid and salmonid fish were held in this system for periods of up to 10 weeks with normal aquarium maintenance procedures.

The following calibration curves were determined using fluorescein dye, where x is the concentration of the tank inflow (%) relative to tank 1 (100%), and y is the peristaltic pump toxicant delivery rate in ml/min. The curves were determined using 2 mm bore diluter tubing with flows of 150 to 250 ml/min.

TANK 2: $y = 68.16 + 0.05.x$

TANK 3: $y = 42.27 + 0.09.x$

TANK 4: $y = 24.33 + 0.11.x$

TANK 5: $y = 9.35 + 0.12.x$

APPENDIX 3

PROBIT ANALYSIS PROGRAM

```
0: wtb 718,27,3,"C3"
1: fxd 3;dim C[36,20];1)r32
2: dim A[4,20];dim B[4,20]
3: "B":fmt 1,2/,"PROBIT ANALYSIS",z;wrt 718.1,"";fmt 2,x,"No.",f3.0
4: wrt 718.2,"",r32;0)r31
5: ent "No.of Doses (<20)?",r1;1)r2
6: fmt 2,/,20x,"Data for Provisional Line",/;wrt 718.2,""
7: fmt 2,4x,"Dose",11x,"N",11x,"Obsd.",7x,"Propn.",5x,"Probit",/
8: wrt 718.2,""
9: for I=1 to r1;ent "Dose=?",A[1,r2]
10: ent "Sample size=?",A[2,r2]
11: ent "No.respondins=?",A[3,r2];if A[3,r2]=0;2.5)r3;sto +3
12: A[3,r2]/A[2,r2])r3)r30
13: cll 'Probitransf'(r3)
14: r3)A[4,r2]
15: fmt 3,2x,f7.2,10x,f3.0,9x,f3.0,10x,fz5.3,7x,f5.3
16: wrt 718.3,"",A[1,r2],A[2,r2],A[3,r2],r30,r3
17: r2+1)r2;next I
18: 1)r2;cfs 1;0)A)B)C)D)E
19: cfs 1;ent "los transformation of dose ?",X;if X=1;sfs 1
20: for J=1 to r1
21: A[1,r2])r3;if fls1=1;los(r3))r3
22: r3)B[1,r2];A[4,r2])r4)B[2,r2]
23: if A[4,r2]=0;sto +2
24: r3+A)A;r3^2+B)B;r4+D)D;r4^2+E)E;r4r3+C)C
25: r2+1)r2;next J
26: (C-AD/r1)/(B-A^2/r1))F
27: D/r1-FA/r1)G
28: ent "Printout working probits etc.??",X;cfs 4;if X=0;sfs 4
29: if fls4=1;sto +2
30: fmt 2,2/,20x,"Computation of weighted line",/;wrt 718.2,""
31: if fls1=1;fmt ,20x,"Los Trans. of x applied";wrt 718,""
32: if fls4=1;sto +3
33: fmt 2,5x,"x",7x,"Prbt Y",5x,"Weight",5x,"Prbt y",8x,"wx",9x,"wy",/
34: wrt 718.2,""
35: "A":1)r2;0)W)r9)r10)r11)r12)r13
36: for K=1 to r1;B[1,r2])r3;A[2,r2])r4;A[3,r2])r5
37: G+Fr3)B[2,r2])r6
38: exp(-.5(r6-5)^2)/\(2{))Z
39: cll 'Revprobit'(r6)
40: r6)P;(Z^2/P(1-P))r4)r7)B[3,r2]
41: B[2,r2]+(r5/r4-P)/Z)B[4,r2])r8
42: r7+W)W;r7r3+r9)r9;r7r8+r10)r10;r7r3^2+r11)r11;r7r3r8+r12)r12
43: r7r8^2+r13)r13
44: r2+1)r2;next K
45: r9/W)r14;r10/W)r15
46: r11-r14r9)r16;r12-r14r10)r17;r13-r15r10)r18
47: r17/r16)F;r14+(5-r15)/F)M)N
48: 1+r31)r31
49: if fls1=1;10^M)N
50: (1/W+(M-r14)^2/r16)/F^2)V
51: \V)S;r18-Fr17)r19;r15-Fr14)G;if fls1=1;10^S)S
52: \(1/r16))r20;1/W)r21
53: 1)r2;if prnd(N,-3)#prnd(0,-3);N)0;sto "A"
54: if fls4=1;sto +5
55: for L=1 to r1;B[3,r2])r7;B[4,r2])r8
56: fmt 5,2x,f7.2,4x,f6.3,5x,f6.3,5x,f6.3,4x,f9.3,2x,f8.3
57: wrt 718.5,"",B[1,r2],B[2,r2],r7,r8,B[1,r2]r7,r7r8
58: r2+1)r2;next L
59: fmt 6,/,5x,"Covariance Matrix",/;wrt 718.6,""
60: fmt 7,5x,"SS(xx)=",f14.3,z;wrt 718.7,"",r16
61: fmt 7,5x,"SS(xy)=",f10.3,z;wrt 718.7,"",r17
62: fmt 7,5x,"SS(yy)",f10.3,z;wrt 718.7,"",r18
63: fmt 8,2/,5x,"REGRESSION LINE",/;wrt 718.8,""
64: fmt 8,5x,"Slope=",f6.3,z;wrt 718.8,F,"";fmt 8,"+-",f6.3;wrt 718.8,"",r20
65: fmt 9,/,5x,"y-Intercept=",f8.3;wrt 718.9,"",G
66: fmt 9,/,5x,"Chi-square for goodness of fit of line=",f7.4,"",z
67: wrt 718.9,"",r19
68: fmt 9,3x,"d.f.=",f3.0;wrt 718.9,"",r1-2
69: ent "Output median,5%&95% EDs???",X;if X=0;sto +15
```

```
70: ent "Input t value for a=.05",r35;5)r36
71: "E":F^2-r35^2r20^2)r37;r14+F(r36-r15)/r37)r34
72: r35\(r20^2(r36-r15)^2+r21r37)/r37)r38
73: r34-r38)r33;r34+r38)r34;if flg1=1;10^r33)r33;10^r34)r34
74: if r36#5;ret
75: fmt 9,/,5x,"Median dose=",f7.3,"+-",f8.3,z;wrt 718.9,"",N,S
76: "F":fmt 9,3x,"95%CL's=",f7.3,x,"&",f8.3;wrt 718.9,"",r33,r34
77: if r36#5;ret
78: 3.3551)r36;r14+(r36-r15)/F)r39;gsb "E"
79: if flg1=1;10^r39)r39
80: fmt 9,/,5x,"5% effective dose=",f8.3,z;wrt 718.9,"",r39;gsb "F"
81: 6.6449)r36;r14+(r36-r15)/F)r39;gsb "E"
82: if flg1=1;10^r39)r39
83: fmt 9,/,5x,"95% effective dose=",f8.3,z;wrt 718.9,"",r39;gsb "F"
84: ent "Store data ?",X;if X=0;gto +3
85: r15)C[1,r32];F)C[2,r32];r14)C[3,r32];r21)C[4,r32];r20^2)C[5,r32]
86: r17)C[6,r32];r16)C[7,r32];W)C[8,r32];1+r32)r32
87: ent "Input more data??",X;if X=1;gto "B"
88: ent "Compare regression lines??",X;if X=0;gto "D"
89: fmt 1,2/,18x,"COMPARISON OF REGRESSION LINES";wrt 718.1,""
90: fmt 1,2/,5x,"Lines",3x,"chi-sq(a)",3x,"chi-sq(b)",6x,"b(comb.)",/
91: wrt 718.1,""
92: "C":ent "First line=??",r3
93: ent "Second line=??",r4
94: C[5,r3]C[5,r4]/(C[5,r3]+C[5,r4]))r5
95: (C[6,r3]+C[6,r4])/(C[7,r3]+C[7,r4]))r6
96: (C[1,r3]-C[1,r4]-r6(C[3,r3]-C[3,r4]))^2)r7;C[7,r3]+C[7,r4])r9
97: r7/(1/C[8,r3]+1/C[8,r4]+(C[3,r3]-C[3,r4])^2/r9))r7
98: (C[2,r3]-C[2,r4])^2/(1/C[7,r3]+1/C[7,r4]))r8
99: fmt 2,4x,f2.0,x,"&",f2.0,4x,f7.3,5x,f7.3,5x,f6.3,"+-",f5.3,/
100: wrt 718.2,"",r3,r4,r7,r8,r6,r5
101: ent "Compare another pair of lines?",X;if X=1;gto "C"
102: ent "Finished????",X;if X=0;gto -15
103: "D":end
104: "Probitransf":p1)p1
105: if p1=1;0)r3;ret r3
106: if p1=0;0)r3;ret r3
107: cfs 3;if p1).5;sfs 3;1-p1)p1
108: \ln(1/p1^2))r4
109: 2.515517+r4(.802853+r4*.010328))r5
110: r5/(1+r4(1.432788+r4(.189269+r4*.001308))))r5
111: r4-r5)r4;if fls3=1;-r4)r4
112: 5-r4)r3
113: ret r3
114: "Revprobit":p1)p1
115: 5-p1)p1;exp(-p1^2/2)/\(2())r20
116: 1-(0>p1))r23;abs(p1))p1
117: 1/(1+p1*.2316419))r21
118: r21(.3193815+r21(-.3565637+1.7814779r21)))r22
119: r22+r21^4(-1.8212559+1.3302744r21))r22
120: r20r22)r20;r20r23+(1-r20)(r23=0))r20
121: r20)r6
122: ret r6
123: end
*24316
```

APPENDIX 4

DATA TRANSFER AND FFT PROGRAMS

1. Data transfer program: PETE

```
      PROGRAM PETE
      DAFIL (10)
      INTEGER R,FS,K,F,L,N,D(1024)
      N=1024
      WRITE(5,15)
15    FORMAT (6X,'NO. OF FILES ? R=',$)
      READ (5,*) R
17    WRITE (5,17)
      FORMAT (/6X, 'DATA TRANSFERRED TO DISK FILE -TEST.DAT-')
      CALL ASSIGN (2,'TEST.DAT')
      DEFINE FILE 2 (0,1024,U,K)
      K=1
      J=-1
18    CALL DATAIN (F,N,D(1),S)
      IF (F-14043) 20,100,100
20    WRITE (5,25) F,K
25    FORMAT (6X,' CASSETTE FILE NO.=',I3,
      1,'- DISK FILE NO.=',I3,' - '$)
      WRITE (2'K) D
      J=J+1
      IF (S-32767) 30,50,50
30    WRITE (5,40)
40    FORMAT (X,'TRANSFER O.K.')
      GOTO 80
50    WRITE (5,55)
55    FORMAT (X,'DATA ERROR')
80    IF (R.EQ.J) GOTO 150
      GOTO 18
100   WRITE (5,110)
110   FORMAT (X,'ERROR IN LEADER')
      GOTO 150
120   WRITE (5,130)
130   FORMAT (X,'ERROR IN DATA')
      GOTO 18
150   END
```

2. Fast Fourier Transfer (FFT) program: **FFT**

```
      PROGRAM FFT

C     This program is a very simple fortran version of the Cooley-
C     Tukey Fast Fourier Transform algorithm. It replaces a set of
C     N complex numbers with the complex valued Discrete Fourier
C     Transform of these numbers.
C     The input is read into the array F so that the real part is
C     in F(1) and the imaginary part is in F(2). The real part of
C     the second number is placed in F(3) and the imaginary part in
C     F(4) and so on. N is the number of complex numbers in the
C     input data and MUST be a power of two.
C     The parameter 'IS' determines the type of transform with
C     IS = +1 producing the so called + root(-1) transform and -1
C     the so-called - root (-1) transform. A +1 transform followed
C     by a -1 transform (or vice-versa) yields the original data
C     multiplied by N.
C     The output is the Power Spectrum which is made up from the
C     sum of the real part squared and the imaginary
C     part squared. The power spectrum has been normalised by dividing by
C     the square of the mean count rate deduced from the
C     input data.
C     The results may be printed or plotted in conjunction with a
C     plot routine.

      DIMENSION F(2048),P(512),IDATA(60)
      LOGICAL*1 FILNAM(20),DATOUT
      REAL*4 IDNAM,DBLK(2),PMIN,PMAX,INTVAL
      INTEGER POINTS,DELTIM,SAMPLE
      EQUIVALENCE (F(1),P(1))
      TYPE 20
20    FORMAT(' ',' FFT PLOT V06-A')
      N=64
      IS=1
      ISPEC=0
      M=0
      DELTIM=0
11    DO 10 I=1,2048
      F(I)=0.
10    CONTINUE
      NT=N
      NIS=IS
      FMEAN=0.
      SIGSQ=0.
      ISAME=0
      IFLAG=0
      DO 12 I=1,512
      P(I)=0.
12    CONTINUE
28    TYPE 21,N
21    FORMAT('0',' How many points ? [',I$,']: '$)
      ACCEPT 22,N
22    FORMAT(I4)
      IF(N.LT.0) GOTO 99
      IF(N.GT.1024) GOTO 50
      IF(N.EQ.0) N=NT
      A=N
23    IF(A-1) 26,25,24
```

```
24    A=A/2
      GOTO 23
26    TYPE 27
27    FORMAT(' ',' Must be a power of 2!!!!')
      GOTO 28
25    NT=N
      TYPE 40,IS
40    FORMAT(' ',' +ve or -ve transform ? [',I2,']: '$)
      ACCEPT 41,IS
41    FORMAT(I2)
      IF(IS.EQ.0) IS=NIS
      IF(IS.EQ.1) GOTO 117
      IF(IS.NE.-1) GOTO 43
117   NIS=IS
66    FORMAT('0',' Input File Name ? '$)
      READ (5,67) IQ,FILNAM
67    FORMAT(Q,20A1)
      IF(IQ.EQ.0) GOTO 99
      CALL FNR50(FILNAM,IQ,DBLK)
      IF(IFETCH(DBLK).NE.0) STOP 'Fetch Error'
      OPEN(UNIT=3,NAME=FILNAM,READONLY,TYPE='OLD')
      READ(3,45) STIME
45    FORMAT(7X,F6.3)
      INTVAL=STIME*SAMPLE
      IF(M.EQ.0) GOTO 76
      DO 68 J=1,M
         READ(3,69) IDUMMY
69       FORMAT(10(5X,I4,1X))
68    CONTINUE
76    L=1
      DO 70 I=1,N*SAMPLE,60
      DO 615 J=1,60,10
      READ(3,69) (IDATA(K),K=J,J+9)
615   CONTINUE
      DO 630 J=1,60,SAMPLE
      DO 620 K=1,SAMPLE
      F(L)=F(L)+IDATA(J+K-1)
620   CONTINUE
      L=L+1
630   CONTINUE
70    CONTINUE
      CALL CLOSE(3)
84    TYPE 82
82    FORMAT('0',' Print Input Data ? '$)
      CALL ANSWER(IN)
      IF(IN-1) 72,85,84
72    TYPE200
200   FORMAT('0',' Plot Input Data ? '$)
      CALL ANSWER(IN)
      IF(IN-1) 203,205,72
205   DO 206 I=1,N
      F(I)=F(I)*5.0/INTVAL
      IF(INTVAL.LT.1.0) F(I)=F(I)/2.0
206   CONTINUE
      DELTIM=0
      POINTS=1024
      IFLAG=1
      GOTO 220
204   DO 77 I=1,N
```

```
      FMEAN=FMEAN+F(I)
77    CONTINUE
      FMEAN=FMEAN/N
      DO 78 I=1,N
      SIGSQ=SIGSQ+(F(I)-FMEAN)*(F(I)-FMEAN)
      F(I)=F(I)-BACK
78    CONTINUE
      SIGSQ=SIGSQ/(N-1)
      PRINT 79,FMEAN,SIGSQ
79    FORMAT('0','<L+B>' = ',F6.1,' Sigma Squared = ',F5.1)
      PRINT 33,INTVAL
33    FORMAT('0',' Sample Integration Time = ',F6.3)
      CALL FORFFT(F,N,IS)
      DO 501 I=1,N/2
501   P(I)=P(I)*P(I)/(FMEAN*FMEAN)
110   TYPE 100
100   FORMAT('0',' Do you want Printout ? : '$)
      CALL ANSWER(IN)
      IF(IN-1) 102,111,110
111   DO 38 I=1,N/2,10
      PRINT 37,I,(P(J),J=I,I+9)
37    FORMAT(' ',I4,2X,':',2X,10F12.1)
38    CONTINUE
99    END
```

APPENDIX 5

"SYNTHESIS OF TETRACHLOROISOPHTHALO-[C^{14}]-NITRILE"

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SYNTHESIS OF TETRACHLOROISOPHTHALO-[^{14}C]-NITRILE

PETER E. DAVIES

Department of Zoology, University of Tasmania,
Box 252C, G.P.O. Hobart, Tasmania, Australia, 7001.

SUMMARY

A low cost, high yield synthesis of tetrachloroisophthalo-[^{14}C]-nitrile (TCIN) is described. Direct replacement of iodine in 3-iodobenzonitrile using ^{14}C-labelled cuprous cyanide, and vapour phase chlorination yields [^{14}C]-TCIN, with maximum utilisation of the radiolabel. Alternative and unsuccessful routes are also briefly described.

KEY WORDS: Tetrachloroisophthalonitrile; Chlorothalonil; ^{14}C label; Synthesis

Tetrachloroisophthalonitrile (TCIN), or Chlorothalonil, is widely used as a broad-spectrum fungicide (1). TCIN labelled with ^{14}C was required for a study of its metabolism and ecological fate. The industrial approach to the synthesis of TCIN is by catalytic ammoxidation of m-xylene and subsequent chlorination, the whole process being performed in the vapour phase (2). This approach, already attempted at laboratory scale (3), is impracticable for the synthesis of [^{14}C]-TCIN due mainly to the high cost of ring-labelled m-xylene.

It is known that TCIN readily undergoes nucleophilic attack at the chlorinated ring carbons adjacent to the nitrile functions (4, 5). Some hydrolysis of the nitrile functions to amide has been reported (5).

Generally, however, the nitrile functions are expected to be biologically stable. Hence, ^{14}C labelling in these functions is a viable low cost route to generating labelled TCIN.

Several routes were attempted. Route 1 (Fig. 1) was unsuccessful due to the failure of formation of the bis-diazonium salt (IV). Hodgson and Heyworth (6) successfully generated isophthalonitrile by the analogous route, but at a yield of only 25.4 %. This was repeated and gave similar but variable yields. Chlorination of sites adjacent to the amine functions appears to inhibit formation of a stable bis-diazonium salt, although the high yield of bis-diazonium salt (82 %) generated from 4,6-diamino-m-xylene and a subsequent Sandmeyer generation of the di-cyano compound (no yield given) seems to preclude a direct steric effect (7).

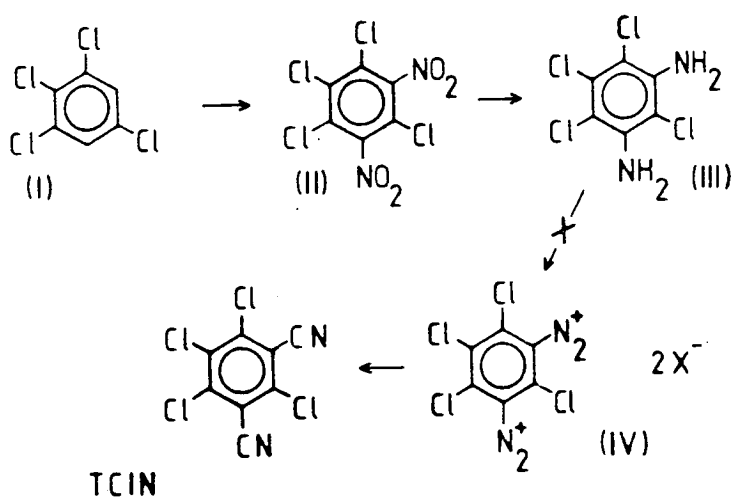

Fig. 1. Route 1

Secondary chlorination of the isophthalonitrile formed by the di-Sandmeyer reaction, as described by Hodgson and Heyworth (6), is possible but radiolabel loss and low reaction yield prohibit the use of this route. Route 2 (Fig. 2) involved an attempt at replacing Br with CN by direct nucleophilic replacement, using CuCN, in 1, 3-dibromotetrachlorobenzene (V).

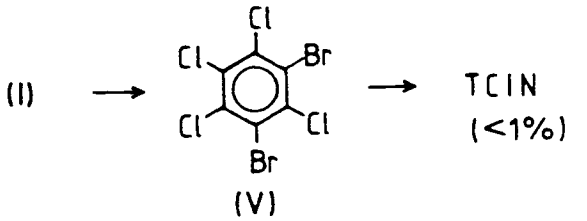

Fig. 2. Route 2

The cyanylation reaction was monitored by gas chromotography (Fig. 3), and showed significant production of the primary replacement product (Fig. 3, Va). However, it appears that the second bromine no longer competes successfully with the remaining chlorines for replacement. Several products are observed, probably with multiple replacement, and only a small fraction of the total is TCIN ($<$ 1 % at 4 h).

Route 3 (Fig. 4) involved replacing halogen (Cl,Br,I) with CN from CuCN (9, 10) in 3-halogenated benzonitrile, and chlorinating in the vapour phase. The first step was successfully carried out for Br and I, with I giving the highest yields (Table 1). Reaction conditions were investigated (10, 11), and both N-methyl pyrrolidone (NMP) and dimethylformamide (DMF) were used as solvents. Catalysis of this reaction type has been reported (12, 13) but was not investigated.

TABLE 1 CN replacement yields

Solvent	Halogen		
	Cl	Br	I
DMF	N.R.	50%	-
NMP	-	65%	92%

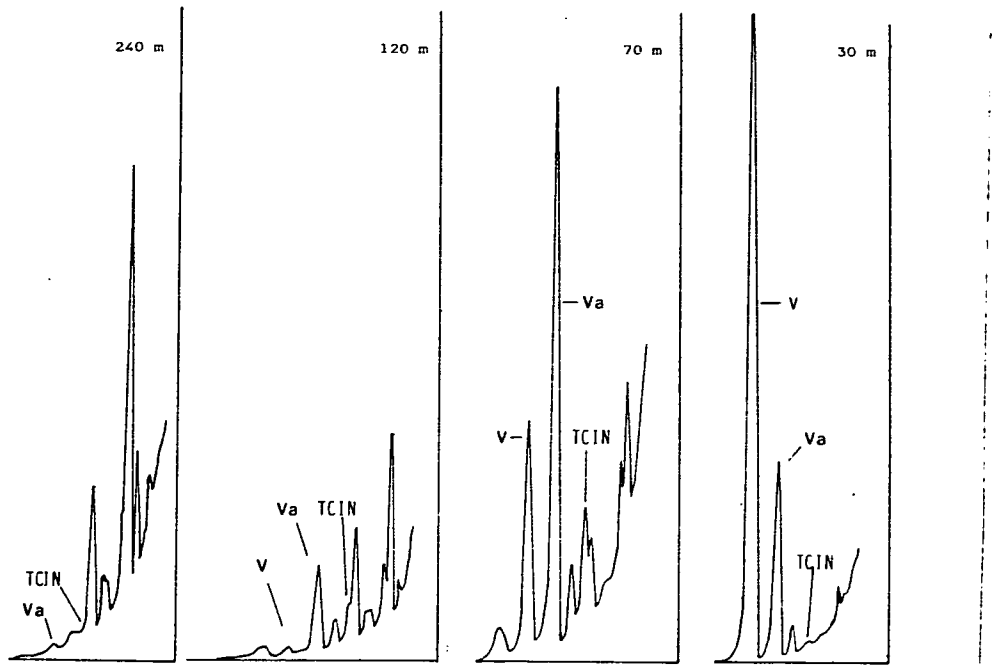

Fig. 3 : Gas chromatograms of the reaction between CuCN and 1, 3-dibromo-tetrachlorobenzene (V) in refluxing DMF at different times (min). Va is the mono replacement product.

Chlorination gave a gradual conversion of isophthalonitrile (VI) to TCIN in the vapour phase at temperatures between 250-300^{o} C with high yields, although some form of catalysis is important (14). Attempts at chlorination in high boiling solvents were unsuccessful.

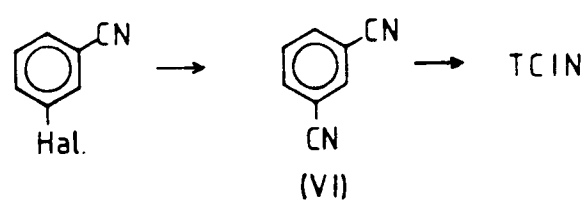

Fig. 4. Route 3

Synthesis of 3-iodobenzonitrile (IX) was as indicated in Fig. 5. CuCN was made fresh and dried in vacuo. After optimization, the reaction was repeated several times with [^{14}C]-CuCN to give [cyano-^{14}C] TCIN with activity of 0.733-0.762 mCi/mmol from [^{14}C]-KCN of activity 57.8 mCi/mmol, which had been diluted with KCN to 1.18 %. This final product activity was 107-112 % of expected.

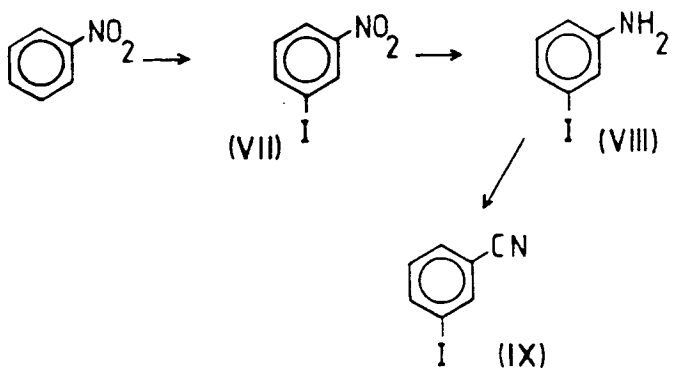

Fig. 5. Synthesis of IX

It should be noted in this context that ^{14}C labelling of CN functions can be carried out by a combination of thermal decomposition and neutron irradiation of Cu_3N (20). Benzo-[^{14}C]-nitrile has been prepared by this route (with 10 % radiochemical yield), although further substitution has not been attempted.

The use of Route 3, Hal. = I, with an overall radiochemical yield of 51 %, from a relatively cheap starting material is recommended for the synthesis of (cyano-^{14}C) TCIN.

EXPERIMENTAL

[^{14}C]-KCN (57.81 mCi/mmol) was obtained from Amersham International, Australia. NMP and DMF were freshly distilled and dried. Melting points were determined on a Yanagimoto Seisakusho micro-melting point apparatus, and are uncorrected. Preparative TLC was performed on Merck silica gel GF254. Infrared spectra were determined as mulls on a Beckman IR-33 spectrometer. N.m.r. (H) spectra were performed at 100 MHz with a Jeol JNM-4H-100 spectrometer, tetramethylsilane as internal standard. E.I. Mass spectra were obtained on a Micromass 7070F double focusing mass spectrometer. G.C. analysis was performed on a Philips PV4000 series gas chromatograph, at conditions indicated, with an SE30 column, nitrogen gas as carrier.

2, 4, 5, 6-Tetrachlorodiaminobenzene (III)

Produced by nitration of 1, 3, 4, 5-tetrachlorobenzene, by the method of Jackson and Carlton (15) with chloroform extraction and recrystallization from Lt. petrol (40-60^{o}); 70 % yield. Quantitative reduction of the dinitro compound gave (III), by the method of Yakobson et al. (16).

Diazonium salt of (III). (IV)

Attempts at diazotisation by the method of Hodgson and Heyworth (6), and at making the fluoroborate salt under anhydrous conditions by the method of Doyle and Bryker (17) were unsuccessful.

1, 3-Dibromotetrachlorobenzene (V)

Synthesized in 90 % yield by the method of Hugel et al. (8), as fine colourless needles, M.P. = 250-253^{o} C.

Cyanylation of (V)

(V) was refluxed in dry DMF with fresh CuCN (1.15 molar ratio). Samples were taken at intervals over 4 h and worked up as in (11), Method C. The dried benzene layers were analyzed by G.C. (210^{o} C), as in Fig. 3.

m-Iodoaniline (VIII)

Nitrobenzene was iodinated according to (18), to give a 47 % yield of an oil, 90% pure by G.C., (VII). Reduction by the method of Yakobson et al. (16) gave (VIII), as an oil, pure by G.C., in 80 % yield.

3-Iodobenzonitrile (IX)

(VIII) was subjected to a Sandmeyer reaction (6) to give 41 % (IX) as colourless needles (M.P. = 39-40^{o} C, lit. 40^{o} C), after preparative TLC (Rf 0.65, Lt. petrol-acetone, 8:1).

Isophthalonitrile (VI)

(IX), 229 mg, was dried in vacuo and dissolved in dry distilled NMP (7 ml). CuCN (produced by method of Chabannes et al. (19), 90 % yield, and dried by azeotropic benzene distillation in vacuo), 104 mg, was added and the whole refluxed under nitrogen for 2.5 h. The mixture was cooled, mixed with hot 30 % NaCN solution, diluted to 100 ml with cold water, and extracted with benzene (3 x 20 ml). The benzene layers were combined, extracted with 10 % NaCN (2 x 50 ml), water (3 x 50 ml) and then dried (Na_2SO_4, anhyd.). The benzene was evaporated to give 150 mg crude material which was subjected to TLC (Lt. petrol-acetone, 8 : 1.5). This gave 118 mg (VI) (92 % yield). M.P. = 162-164^{o} C (lit. 162^{o} C). M^{+} = 128. N.m.r. ($CDCl_3$), 7.6-8.1, multiplet.

The above reaction was performed with [^{14}C]-KCN, diluted to 1.18 % by KCN, to produce [^{14}C]-CuCN (90 % yield) and isophthalo-[^{14}C]-nitrile in 75 % yield.

Tetrachloroisophthalonitrile

(VI), 100 mg, was dried in vacuo and introduced into a 100 ml thick-wall, round flask with a Rotaflo $^{(R)}$ tap adaptor, with 400 mg of preactivated charcoal as catalyst.

Fine charcoal was preactivated by heating in the above bulb filled with chlorine gas at 220^{o} C for 2 h. After evacuation and refilling with chlorine the bulb was sealed and heated at 250-300^{o} C by immersion in a Woods metal bath for 4.5 h. Cooling, evacuation, acetone extraction (3 x 20 ml) gave, on filtering, evaporation and vacuum drying, 160 mg of TCIN (95 %; 75 % yield). G.C. analysis was performed on cooled flask wall deposits during a trial run.

The same yield was obtained on performing the reaction with isophthalo-[^{14}C]-nitrile from the previous reaction. Radioactivity = 0.733-0.762 mCi/mmol; expected = 0.705 mCi/mmol. TCIN produced by this method, was identical by M.P., TLC, G.C. and M.S. with purified technical material.

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